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Expression Of Human Kallikrein Protein And mRNA In Maxillofacial Cysts And Tumours

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology

EXPRESSION OF HUMAN KALLIKREIN PROTEIN AND mRNA IN MAXILLOFACIAL CYSTS AND TUMOURS

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Abstract

Non-inflammatory odontogenic cysts and tumours vary in clinical appearance and can cause widespread destruction and deformation of the face. Proteases may play a role in the differing pathogenesis of these cysts and tumours. Human kallikrein proteins (KLKs) are a group of 15 serine proteases implicated in a variety of signalling and regulatory roles. This study evaluated the ameloblastoma, keratocystic odontogenic tumour (KOT), dentigerous cyst, lateral periodontal cyst, nasopalatine duct cyst and odontoma for the presence of KLK 3, 4, 5, 9 and 11 utilizing immunohistochemistry. Secondarily, we evaluated ameloblastoma for expression of KLK 1-15 mRNA using reverse transcription-polymerase chain reaction. Immunostaining identified KLK 3, 4, 9 & 11 in all tissue types and KLK 5 only in KOT's. The ameloblastoma expressed KLK 1, 4, 7, 8, 10 & 12 mRNA. For the first time KLK 3, 4, 5, 9 & 11 were identified in maxillofacial cysts and tumours.

Keywords

odontogenic tumours, odontogenic cysts, oral and maxillofacial pathology, head and neck pathology, human kallikrein proteins, kallikrein protein, biomarkers, prostate specific antigen, immunostaining, protein expression, RNA expression

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List of Abbreviations

BCL-2: B Cell Lymphoma-2 Protein

BCL-X_L: B Cell Lymphoma–X_L Protein

CA-125: Cancer Antigen 125

CEJ: Cemento-enamel junction

CN: Cranial Nerve

cDNA: Complementary Deoxyribonucleic Acid

DAB: Diaminobenzidine

DNA: Deoxyribonucleic Acid

ECM: Extracellular Matrix

FFPE: Formalin Fixed Paraffin Embedded

FGF: Fibroblast Growth Factor

IGF: Insulin Like Growth Factor

IL-1: Interleukin 1

IL-6: Interleukin 6

KLK: Human Tissue Kallikrein

KLK: Human Tissue Kallikrein Gene

KOT: Keratocystic Odontogenic Tumour

MMP: Matrix Metaloproteinase

mRNA: Messenger Ribonucleic Acid

NOS: Not Otherwise Specified

OSS: Overall Staining Score

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PHRP: Parathyroid Hormone Related Peptide

PTCH1: Patched 1 Tumour Suppressor Gene

PSA: Prostate Specific Antigen

RNA: Ribonucleic Acid

RT-PCR: Reverse transcription – polymerization chain reaction

TGF- β: Transforming Growth Factor Beta

TNF-α: Tumour Necrosis Factor Alpha

Chapter 1

1 Embryology of the Oral and Maxillofacial Complex

Knowledge of the formation of the tissues comprising the face, mouth and jaws is essential in order to understand the pathogenesis of developmental oral and maxillofacial lesions. Despite the complexity and large breadth of this topic, a concise review will be presented in this chapter in order to provide a foundation for the subsequent discussion of various odontogenic and non-odontogenic pathologies.

1.1 The Developing Maxillofacial Complex

The first three weeks of embryonic development yield a three layered embryo composed of ectoderm, mesoderm and endoderm. Over the next seven weeks, developmental changes will occur forming the basis for the major structural components of the head and neck.

Folding of the trilaminar embryo at approximately three weeks results in formation of the stomodeum, an ectodermal lined primitive oral cavity separated from the gut by the buccopharyngeal membrane. The buccopharyngeal membrane is a bilayered structure composed of ectoderm and endoderm which will eventually break down, facilitating a connection between the oral cavity and gut. Lateral to the stomodeum are the branchial arches, structures of neural crest origin. Neural crest cells arise from the lateral aspect of the neural plate, as it folds to form the neural tube. These neural crest cells undergo an epithelial to mesenchymal transition, and subsequent migration away from the neural tube (1). Tissues derived from the neural crest (including cranial bone, alveolar bone, dentin and periodontal ligament) are considered ectomesenchymal in origin, reflecting the neuroectodermal embryologic basis (2). Development of the neural crest is a crucial step in formation of a normal anatomic maxillofacial complex. Abnormalities ranging from alterations in dental development, to craniofacial syndromes such as Treacher-Collins syndrome may result due to improper development of the neural crest.

Five paired branchial arches (numbered I-IV and VI) are oriented in a rostral-caudal relationship form along the lateral walls of the developing pharynx. Branchial grooves separate the arches laterally, while pharyngeal grooves separate the arches medially. Growth of these arches separates the stomodeum from the cardiac bulge (2). Branchial arches are composed of an outer ectodermal surface, inner endodermal surface, and central core composed of mesoderm and ectomesenchyme which gives rise to cartilage in each arch. The first branchial arch forms rostrally to the buccopharyngeal membrane; as a result it has an ectodermal inner aspect as opposed to the endodermal inner aspect as found in the remaining four arches. Each arch has a vascular and nerve supply consisting of sensory and motor branches(2). Anatomic muscular, skeletal and nervous derivatives from each branchial arch are noted in Table 1 (3).

Table 1.1: Branchial Arch Derivatives

Arch Number	Muscle(s)	Nerve	Skeletal
I	Anterior belly of digastric, mylohyoid, tensor tympani, tensor palatini, temporalis, masseter, medial pterygoid, lateral pterygoid	Trigeminal (CN V)	Meckel's Cartilage: mandible, maxilla incus, malleus, sphenomandibular ligament, sphenomalleolar ligament
II	Muscles of facial expression, stapedius, stylohyoid, posterior belly of digastric	Facial (CN VII)	Reichert's Cartilage: Hyoid bone (lesser cornu, upper part of body), stapes, styloid process of temporal bone, stylohyoid ligament,
III	Stylopharyngeus	Glossopharyngeal (CN IX)	Hyoid bone (greater cornu, lower part of body)
IV	Pharyngeal constrictors, laryngeal musculature	Vagus (X)	Laryngeal cartilage
VI	Sternocleidomastoid, trapezius	Spinal Accessory (XI)	

Initially, the stomodeum is bounded rostrally by the first branchial arch's frontal prominence, and caudally by the cardiac bulge. Maturation and development of the branchial arches results in the first, second and third branchial arches forming the floor of the stomodeum as the cardiac bulge is displaced caudally. Between three to four weeks, the first branchial arch forms bilateral (lateral) maxillary processes and (inferior/caudal) mandibular processes which enclose the stomodeum. The frontal prominence gives rise to the medial nasal process, lateral nasal process and nasal pits in the fourth to fifth weeks. Development of the external face during week five to six results in medial ingrowth of the maxillary processes, resulting in fusion of the right and left medial nasal processes at the midline, and ultimately the maxillary processes with the central medial nasal processes. As the maxillary/midface development occurs, the mandibular processes fuse in the midline forming the lower lip. Fusion of the aforementioned facial processes during weeks four to six establishes the support and structure required for normal odontogenesis. Areas of odontogenic epithelium arise from thickening of the epithelium along the inferior border of the maxillary process as well as the superior border of the mandibular process as early as week four. Once these facial processes fuse (typically by day thirty-seven), a continuous odontogenic epithelial thickening known as the primary epithelial band is formed in the primitive maxilla and mandible (2).

1.2 Odontogenesis

Odontogenesis, the formation of teeth, is a complex process involving maturation, morphogenesis and histodifferentiation of the maxillary and mandibular primary epithelial bands. The dental lamina and vestibular lamina arise from the primary epithelial band. The vestibule arises due to ingrowth of the vestibular lamina into the underlying ectomesenchyme. Prior to vestibular lamina ingrowth, the dental lamina forms as placodes, areas of thickening of the epithelial band form along the primary band. These epithelial placodes will grow/bud into the underlying mesenchyme, signaling the mesenchyme to condense around them (4). Normal tooth development occurs in three classic stages following the aforementioned events of initiation. The bud, cap and bell stages constitute morphogenesis in tooth development (4). Differentiation begins to occur in the late bell stage, and continues throughout development yielding mature teeth.

1.2.1 Bud Stage

The bud stage is characterized by the invagination of the primary epithelial band into the ectomesenchyme. As mentioned, the dental lamina is the epithelial connection between the oral epithelium and the newly formed tooth bud. Ectomesenchymal tissue in the vicinity of the epithelial bud condenses in response to the bud ingrowth. The transition from bud to cap stage is the initiation of the morphologic difference between tooth buds, ultimately leading to different shapes of teeth (2).

1.2.2 Cap Stage

Odontogenesis continues with further growth of each bud into the underlying connective tissue, 'dragging' the dental lamina with it. Once an appropriate depth is reached, the basal cells at the tip of the bud proliferate and extend inferiorly and laterally forming a concavity, the early cap stage of odontogenesis (5). Continued development of the cap stage results in a separation of the top and bottom layers of odontogenic epithelium, forming the outer enamel epithelium (superficial layer) and inner enamel epithelium (deep layer). This developing structure is referred to as the enamel organ (2). As the distance between the inner and outer enamel epithelium increases, the space is occupied by an increasing number of star shaped epithelial cells known as the stellate reticulum. Glycosaminoglycans secreted into this central space (between epithelial layers) lead to water inflow and increasing space between the epithelial cells (2).

The cap stage is named as a result of the shape of the enamel organ which overlies the condensed ectomesenchyme/dental papilla. Ectomesenchyme surrounding the periphery of the cap/enamel organ/epithelium is termed the dental follicle. The dental follicle will form marginal gingiva as well as the periodontal ligament, connecting cementum of the tooth to alveolar bone (5). Further elongation at the periphery of the cap, and histodifferentiation of cells of epithelial and ectomesenchymal origin leads to the next stage of development, the bell stage.

1.2.3 Bell Stage

The bell stage is characterized by continued growth, morphodifferentiation (forming the shape of the crown of the tooth) and histodifferentiation of the inner enamel epithelium cells into elongated, columnar, palisaded cells with nuclei located opposite the underlying connective tissue/basement membrane. This reverse polarization identifies the differentiation of these cells into immature ameloblasts (enamel forming cells). Bell stage inner enamel epithelium induces the surrounding ectomesenchymal connective tissue to change form, ultimately resulting in the mature dental papilla, tissue that will become the future pulp of the tooth. In response to the reverse polarization of the immature ameloblasts, ectomesenchymal cells adjacent to the ameloblasts differentiate into a layer of palisaded immature odontoblasts which oppose the ameloblast basement membrane.

Inner enamel epithelium/ameloblasts and the outer enamel epithelium maintain continuity at the deepest aspect of the developing bell. The cervical loop is the point at which the concavity of the bell has a point of inflexion and transition from inner enamel epithelium/ameloblast to outer enamel epithelium occurs. A new layer of cells forms via differentiation from the stellate reticulum. This layer, the stratum intermedium assists in production of enamel and lies between the stellate reticulum and inner enamel epithelium. Late bell stage is characterized by secretion of the dentin matrix by odontoblasts, which subsequently stimulates secretion of enamel matrix by the ameloblasts (5).

1.2.4 Late Odontogenesis

Above the developing tooth, dental lamina forms small, discontinuous islands of residual epithelium in the connective tissue. These are termed the cell rests of Serres. Ameloblasts/outer enamel epithelium remaining after tooth formation constitutes the reduced enamel epithelium, a layer of epithelial cells surrounding the crown of the tooth. Odontoblasts at the deep aspect of the bell, away from the oral cavity are responsible for dentin matrix production for formation of the tooth root. Dentin production induces adjacent dental follicle tissue to differentiate into cementoblasts, the cells responsible for

production of cementum, the outer component of the root. Upon completion of dentin formation, these odontoblasts (forming the Hertwig's root sheath) fragment into islands of epithelial cells known as the cells rests of Malassez. Cell rests of Malassez remain in the periodontal ligament (if present) throughout life (5).

Chapter 2

2 Oral and Maxillofacial Pathology

Oral and maxillofacial pathology is the broad area of study and investigation of developmental, genetic, acquired or functional abnormalities of the oral and maxillofacial region. Medical and dental professionals including specialists and generalists deal with pathology in the mouth, face, jaws and neck on a regular basis. Odontogenic tissues lead to a small but unique subset of head and neck disease as they may involve multiple embryologic tissues in one pathologic process. These diseases/disorders may be benign or malignant, with significant variation in prognosis and treatment.

2.1 Non-Inflammatory Odontogenic Cysts

Epithelial lined cysts within bone are rare apart from the oral and maxillofacial region (6). Odontogenic cysts can be classified as neoplastic, developmental or inflammatory. Inflammatory odontogenic cysts including the radicular cyst and buccal bifurcation cyst form as a result of inflammation, often caused by pulpal disease of the tooth. Developmental odontogenic cysts are of unknown cause, and can form anywhere that odontogenic tissue is/has been present. Examples of developmental odontogenic cysts are the dentigerous cyst, lateral periodontal cyst, eruption cyst, gingival cyst of the adult, glandular odontogenic cyst and the orthokeratinizing odontogenic cyst. Cystic neoplasms include the keratocystic odontogenic tumour and the calcifying cystic odontogenic tumor.

The clinical presentation, radiographic appearance and histologic features of these lesions are quite variable. Non-inflammatory odontogenic cysts may present as radiographic change visible on routine dental imaging. Rarely, they may present as a result of a pathologic fracture of bone through the cystic structure. Odontogenic cysts should be considered in the differential diagnosis for expansile lesions of the jaws, as well as non-expansile radiolucencies on routine radiographic examination. A discussion of the clinical behavior, histology and treatment of odontogenic cysts studied as part of this project is included in the following text.

2.1.1 Dentigerous Cyst

The dentigerous cyst is the most common non-inflammatory odontogenic cyst, representing 20% of all of these lesions (6). Dentigerous cysts occur in association with an unerupted tooth, most commonly associated with mandibular third molars. A smaller proportion are associated with maxillary third molars, maxillary canines and mandibular premolars (7). Deciduous teeth are very rarely associated with dentigerous cysts. There is a male predilection, with common age of presentation ranging from the teens to fifth decade of life (6, 8). Fluid accumulation between the reduced enamel epithelium and enamel of the affected teeth is believed to account for the formation of these cysts (7). Dentigerous cysts present in a variety of scenarios ranging from small, painless cysts with minimal bony destruction to large cysts with bony expansion. They may become infected when occurring with a partially erupted tooth, or as a result of association with a periodontal or pericoronal lesion of an adjacent tooth resulting in inflammatory change around the cyst, pain and potentially suppuration (6).

Radiographically, these cysts typically present as a unilocular radiolucency with a well-defined sclerotic border arising from the cemento-enamel junction (CEJ) of the affected impacted tooth. The crown of the impacted affected tooth is surrounded by the cyst in the central dentigerous cyst (described above). The lateral dentigerous cyst presents with the cyst developing laterally along the root surface enclosing one side of the root and the crown of the tooth. The very rare circumferential variety of dentigerous cyst is present when it appears that the entire root and crown of the tooth are enveloped by the cyst (7). It is possible to have displacement of the associated tooth as a result of the dentigerous cyst's growth. If displacement occurs, the mandibular third molar may be displaced to the inferior border of the mandible, or superiorly into the ramus of the mandible (6). Radiographic studies are inadequate to confirm a diagnosis of dentigerous cyst. Small cysts and hyperplastic dental follicles may present with similar radiographic features. As a result, pericoronal tissue removed with impacted wisdom teeth should be submitted for histopathologic analysis if a pre-operative radiolucency of greater than about 5 mm is detected (7) (9).

Histologically the dentigerous cyst is composed of a non-keratinized stratified squamous epithelial. The presence of nodular epithelial thickenings has been noted and must be examined to rule out neoplastic change (6). There is often a thin fibrous connective tissue wall composed of immature fibroblasts separated by ground substance and a stroma rich in mucopolysaccharides (10). If inflamed, the connective tissue wall will exhibit more collagen and an infiltration of inflammatory cells.

Although reports exist describing the transition of dentigerous cyst to squamous cell carcinoma, ameloblastoma or intraosseous mucoepidermoid carcinoma, the occurrence is rare (6). Treatment of dentigerous cysts usually involves surgical enucleation with extraction of the associated tooth. Large dentigerous cysts may be treated with marsupialization and subsequent enucleation of a smaller cyst, resulting in a smaller bony defect (7, 11). Recurrence after enucleation is rare.

2.1.2 Lateral Periodontal Cyst

The lateral periodontal cyst is a developmental odontogenic cyst that usually occurs lateral to the root surface of a tooth, most commonly arising in the mandibular premolar region while in the maxilla, they often occur between the canine and lateral incisor teeth (6). Gingival cysts of the adult are histopathologically similar to the lateral periodontal cyst but instead of presenting as an intrabony cyst, are found in the gingiva. Lateral periodontal cysts are relatively rare, constituting less than 2% of epithelial lined maxillofacial cysts (6). Remnants of the dental lamina within bone are believed to give rise to the lateral periodontal cyst (11). Lateral periodontal cysts most commonly present between the fifth and seventh decades of life with a 2:1 male:female predilection (11).

Lateral periodontal cysts are often asymptomatic and detected as a result of routine dental radiography. Radiographically, these cysts are usually well defined, round or tear-dropped shaped radiolucencies presenting lateral to the roots of premolar or canine teeth. Lateral periodontal cysts are not commonly associated with tooth root divergence or root destruction (11). Lesions greater than 1 cm in diameter are rare. Occasionally a polycystic radiographic appearance may be present consistent with the histopathologic diagnosis of a botryoid odontogenic cyst (6).

Diagnosis of a lateral periodontal cyst is based on histopathologic examination. Inflammatory cysts such as a radicular cyst from an accessory root canal may appear with similar radiographic presentation to the lateral periodontal cyst. Histopathologic characteristics of the lateral periodontal cyst include a fibrous wall with thin squamous or cuboidal non-keratinized epithelial lining (one – three cells thick in most areas) with or without the presence of nodular focal thickenings composed primarily of clear cells (6). Botryoid odontogenic cysts may present with multiple lateral periodontal cysts and focal areas similar to that seen in glandular odontogenic cysts (6).

Surgical curettage and enucleation are the treatment of choice for lateral periodontal cysts. Recurrence is rare, however a higher risk of recurrence is noted for the botryoid odontogenic cyst (11).

2.2 Benign Odontogenic Tumours

Benign odontogenic tumours may originate from epithelial tissue, ectomesenchymal connective tissue or a combination of both (mixed tumours). These tumours originate in the mandible or maxilla, and form from tissue required for normal odontogenesis. Examples of epithelial odontogenic tumours are the ameloblastoma, keratocystic odontogenic tumour, calcifying epithelial odontogenic tumour and adenomatoid odontogenic tumour. Connective tissue/mesenchymal tumours include the odontogenic fibroma, odontogenic myxoma and the cementoblastoma. Mixed (both epithelial and mesenchymal/connective tissue origin) odontogenic tumours include the ameloblastic fibroma, and ameloblastic fibro-odontoma.

A review of the ameloblastoma and keratocystic odontogenic tumour will be presented in this chapter.

2.2.1 Ameloblastoma

The ameloblastoma is a benign odontogenic neoplasm originating from odontogenic epithelial cells in the jaws/mouth. Ameloblastomas are slow growing, locally aggressive tumours with limitless growth potential and a high rate of recurrence. If left untreated they may cause significant destruction of facial form, including

compression of vital structures such as nearby vasculature and nerves. These tumours are the second most common clinically significant benign epithelial odontogenic tumour (following the keratocystic odontogenic tumour) (6, 7). Sources of the neoplastic epithelial cells in ameloblastomas include the oral epithelium, cell rests of Serres, cell rests of Malassez, lining of odontogenic cysts and the reduced enamel epithelium (11). The pathogenesis of the ameloblastoma is partially attributable to a number of known altered protein levels. Overexpression of the anti-apoptotic proteins B cell lymphoma-2 protein (BCL-2) and B cell lymphoma–X_L protein (BCL-X_L), tumour necrosis factor alpha (TNF-α), fibroblast growth factor (FGF), interleukin 1 (IL-1), interleukin 6 (IL-6) and matrix metaloproteinases (MMP) are identified in ameloblastoma tissue. Expression of altered laminin 5 has been identified at the epithelial interface (7, 11). Ameloblastomas also show a low cell proliferation rate based on staining for the protein Ki-67 (11). Despite this knowledge about altered levels of protein such as growth factors, cell signaling molecules and cytokines, knowledge regarding a stimulus for neoplastic transformation of the odontogenic epithelial cells remains unknown (11). Although metastasis has been reported, it is extremely rare, reinforcing its classification as a benign neoplasm.

Ameloblastomas are classified based on their histologic characteristics and general clinical form. Three major classifications clinically are identified: the conventional solid/common/multicystic/polycystic ameloblastoma, the unicystic ameloblastoma and peripheral (extraosseous) ameloblastoma.

The solid/common/multicystic/polycystic ameloblastoma is the most prevalent type of ameloblastoma comprising approximately 86% of all ameloblastomas (6). It presents most often in the posterior mandible of patients aged 20 – 70. No gender or racial predilection is noted. These slowly growing tumours may present asymptomatically on routine dental radiography or with painless bony expansion and/or displacement of teeth in the affected area of bone. Radiographically these tumours appear as well defined radiolucencies, often multilocular in nature. Buccal and lingual cortical expansion or perforation are often seen, and destruction of tooth roots in the vicinity of the lesion is common (7). Differential diagnosis based on clinical and

radiographic examination includes other odontogenic and non-odontogenic cysts and tumours. As a result, histopathology is required for diagnosis. Six histologic subtypes of the solid/multicystic ameloblastoma are recognized. These include the follicular, plexiform, acanthomatous, granular cell, desmoplastic and the basal cell types (6, 7). Follicular and plexiform subtypes are the most common while the basal cell variant is the least common (7). The follicular pattern exhibits reverse polarized peripheral columnar epithelial cells, surrounding a stellate reticulum like core. Plexiform variant tumours have anastomosing cords of odontogenic epithelium with reverse polarized ameloblast-like cells lining the periphery of these cords. The solid ameloblastoma tends to infiltrate between intact cancellous bony trabeculae beyond the radiographic border of the tumour (12). Radiographically evident bony destruction is not seen at the margin of tumour invasion. Treatment by simple enucleation and curettage results in recurrence rates ranging from 50-90% (6). As a result, osseous resection 1.0-1.5 cm beyond the radiographic margin is recommended. Soft tissue should be resected such that one uninvolved tissue plane is included in the resection margin (7).

The unicystic ameloblastoma represents roughly 13% of ameloblastomas. It is more commonly seen in younger patients with an average age of presentation of 22.1 years (7). Fifty percent of unicystic ameloblastomas present during teenage years (6). Unicystic ameloblastomas present in the mandible in 90% of cases, often as a painless swelling which surrounds the crown of an unerupted tooth. Radiographic presentation is usually a well-defined, corticated radiolucency which may be confused with the presentation of a dentigerous cyst, keratocystic odontogenic tumour, residual or radicular cyst (depending on location) (6). Three histologic variants are identified. These include the luminal, intraluminal and mural unicystic ameloblastomas. Luminal unicystic ameloblastomas have tumour cells located only in the luminal aspect of the cyst. They are surrounded by a fibrous wall and the cystic lining is composed of reverse polarized, palisaded columnar or cuboidal hyperchromatic basal cells (6). Intraluminal unicystic ameloblastomas exhibit intraluminal masses/nodules of ameloblastic cells often with the plexiform pattern seen in solid ameloblastomas. Inflammation may also be seen in the intraluminal aspect of the cyst. In the mural unicystic ameloblastoma, invasion of islands of epithelial cells resembling plexiform or follicular solid ameloblastomas are seen in the

fibrous wall of the cyst (6). Treatment of the unicystic ameloblastoma is dependent on a number of factors including tumour size, location and histologic subclassification. Intraluminal and luminal unicystic ameloblastomas can be successfully treated with enucleation and curettage. The rate of recurrence after enucleation and curettage of these tumours is 10-30% (7, 13). Mural unicystic ameloblastomas should be treated similarly to the solid ameloblastoma, with a 1.0-1.5 cm bony resection margin as well as inclusion of one uninvolved tissue plane for soft tissue management (7). Block resection as described for the mural unicystic ameloblastoma is also indicated for recurrent intraluminal or luminal unicystic ameloblastomas, as well as for very large unicystic ameloblastomas that occupy such significant portions of the jaw that enucleation would leave little to no bony structure remaining (7).

The peripheral ameloblastoma is the least common variant, accounting for about 1% of all ameloblastomas. The average age of presentation is 52, with most tumours presenting in middle aged adults (6). Clinical presentation is usually a non-ulcerated, sessile or pedunculated lesion of the gingiva or alveolar mucosa, consistent with the presentation of an oral epulis. Peripheral ameloblastomas do not invade bone, but may cause superficial bony erosion (7, 13). Histologically, these tumours may present with the patterns described for any of the solid ameloblastomas, most commonly resembling the plexiform or follicular variants (6). It is possible for the tumour to be sub-epithelial, or in communication with the mucosal/gingival epithelium. Treatment for peripheral ameloblastomas involves wide local excision. If margins are negative, recurrence is rare (7). Malignant transformation has been noted, but is quite rare (6).

2.2.2 Keratocystic Odontogenic Tumour

The keratocystic odontogenic tumour (KOT) is a cystic neoplasm of odontogenic origin. Previously known as the odontogenic keratocyst, it is now classified by the World Health Organization as a benign odontogenic neoplasm as a result of mutations identified in the patched 1 (PTCH1) tumour suppressor gene (14, 15). While odontogenic cysts of the jaws and oral cavity are generally believed to enlarge as a result of osmotic pressure within the cyst lumen, the KOT enlarges as a result of intrinsic mitotic growth of the tumour cells. KOTs express bone resorptive cytokines/factors interleukin-1 (IL-1), IL-6,

and parathyroid hormone related peptide (PHRP) (7). Proliferating cell nuclear antigen (PCNA) has also been identified in KOTs and Slootweg (16) identified increased proliferative activity in KOTs compared to other odontogenic cysts. (KOT growth can also be attributed to mutations in the PTCH1 tumour suppressor gene, which usually functions as a membrane receptor, forming a complex with the sonic hedgehog protein to control growth and cell division). When mutated, the PTCH1 gene fails to suppress tumour growth, resulting in enlargement and expansion of the KOT. The PTCH1 gene and KOTs are associated with the nevoid basal cell carcinoma syndrome/Gorlin-Goltz syndrome. Gorlin-Goltz syndrome has a prevalence of 1 in 60,000 (6). It exhibits autosomal dominant inheritance, however 35-50% of cases are due to de novo mutations (6). The most prominent findings (present in >50% of patients) in Gorlin-Goltz syndrome include multiple basal cell carcinomas, multiple KOTs, epidermal cysts, palmar/plantar pitting, bifid ribs, calcified falx cerebri, ocular telorism, frontal bossing with enlarged head circumference and spina bifida occulta of cervical or thoracic vertebrae (6). KOTs in patients with nevoid basal cell carcinoma syndrome/Gorlin-Goltz syndrome often exhibit satellite cysts and islands of epithelial rests within the cystic capsule. Compared to non-syndromic KOTs, syndromic KOTs show higher levels of tumour suppressor p53 and cyclin-D1(6). Syndromic patients with KOTs should be followed for recurrence. Due to the higher risk of KOTs in these patients, infected KOTs in patients with Gorlin-Goltz syndrome are relatively common compared to the non-syndromic population (6).

KOTs occur in patients of all ages, most commonly presenting in those aged 10 – 40 (6). Roughly 5% of all patients presenting with KOTs will have multiple lesions and an additional 5% will present with Gorlin-Goltz syndrome (11). A small male predilection is noted, and 60-80% of cases involve the mandible, most commonly the ascending ramus and posterior body (7). They are hypothesized to form from cells of the dental lamina, so can form anywhere odontogenesis occurs (6). KOTs may also arise in the mucosa as a result of daughter cysts located between an old cyst and the mucosa (17). Radiographic presentation includes unilocular or multilocular radiolucencies with well-defined corticated borders. They may present with an unerupted tooth involved with the

tumour. A clinical differential diagnosis would commonly include an ameloblastoma, dentigerous cyst, and odontogenic myxoma.

Histopathologic diagnosis is made based on microscopic findings including a cyst usually 6-8 cells thick, with a hyperchromatic palisaded basal layer of cuboidal or columnar epithelial cells. A corrugated but flattened parakeratin layer is noted on the luminal aspect of the cyst. Occasionally, focal areas of orthokeratin are noted in the luminal lining. Commonly, the flat basal layer may detach from its hemidesmosomal attachment to the underlying connective tissue. The cyst lumen is commonly filled with keratin debris on microscopic examination (6), clinically appearing as a cream of wheat like solution which is visible if the cyst lining is ruptured on surgical removal.

KOTs have a relatively high rate of recurrence following surgical enucleation, with studies noting a range of 5-62% (6). The thin cyst lining which is easily ruptured on enucleation as well as propensity for satellite cysts are factors believed to contribute to the high rate of recurrence. As a result, multiple surgical procedures have been described for treatment of the KOT ranging from simple surgical enucleation to block resection. Marsupialization or decompression followed by enucleation with peripheral ostectomy has been shown to reduce the rate of recurrence while minimizing the risk of neurosensory deficit or pathologic fracture when dealing with mandibular KOTs (17).

2.3 Odontogenic Hamartomas

Hamartomas are developmental malformations of normal tissue. Odontogenic hamartomas represent the most common odontogenic tumour; the odontoma is more common than all other odontogenic tumours combined (6). Odontogenic hamartomas are comprised of normal odontogenic tissue; enamel, dentin, pulp and cementum.

2.3.1 Odontoma

Fully developed odontomas consist primarily of dentin and enamel, remaining stable in size and may erupt into the oral cavity (8). In early stages of development odontogenic epithelium and mesenchyme remain surrounding the odontomas. Odontomas are described as compound (multiple small toothlets) or complex (mass of enamel and dentin, not in the shape of a tooth) (6). Largely asymptomatic, odontomas are often found incidentally in the first two decades of life. Odontomas may present with an unerupted tooth, or may be located between roots of teeth (6).

Histopathology of odontomas includes enamel and dentin, with variable amounts of pulp and cementum. Multiple compound odontomas may present in a surrounding fibrous stroma. Immature odontomas may present with odontogenic epithelium and ectomesenchymal tissue surrounding the calcifying odontoma (6). Epithelial rests may remain in the nearby tissue even after complete development of the odontoma. Odontomas may be removed surgically when indicated.

2.4 Non-Odontogenic Maxillofacial Cysts

Non-odontogenic cysts of the head and neck fall primarily into the classification of developmental cysts of the head and neck. These epithelial lined fluid filled cavities increase as a result of increased hydrostatic luminal pressure (6). While a number of non-odontogenic cysts of the head and neck exist, only the nasopalatine duct cyst was studied in this project, and as a result, will be discussed below.

2.4.1 Nasopalatine Duct Cyst

The nasopalatine duct cyst, also known as the nasopalatine canal cyst or incisive canal cyst is the most common non-odontogenic cyst of the maxillofacial region, occurring in 1% of the population (6). This developmental cyst forms in the incisive canal which is derived from the embryologic fusion of the premaxilla with left and right palatal processes (11). Incisive canal contents include the nasopalatine nerve, sphenopalatine and descending palatine arteries. In normal development, the epithelial cells lining the nasopalatine duct degenerate, however in situations with incomplete degeneration, epithelial remnants may give rise to the a nasopalatine duct cyst (6). Possible etiologies of nasopalatine duct cyst development include spontaneous cystic degeneration of contents of the nasopalatine duct, retention of mucous from minor salivary glands, trauma and infection of the duct (6, 11).

Most commonly presenting in the 4th to 6th decades of life, nasopalatine duct cysts are often discovered as incidental findings on routine intraoral radiography. Alternatively, they may present as a swelling of the anterior maxilla/palate with or without pain and drainage (6). The radiographic appearance consists of a well defined radiolucency located between the maxillary central incisors.

Histopathology is variable, but diagnosis is made based on the presence of pseudostratified ciliated columnar (respiratory) epithelium, simple columnar, cuboidal and/or stratified squamous epithelium with or without neurovascular bundles and mucous glands in the cyst wall (6, 8). Acute or chronic inflammation may be present in the cystic wall. Malignant transformation has been reported but is very rare. Surgical enucleation rarely results in recurrence.

Chapter 3

3 Kallikreins

The past 30 years have resulted in a rapid expansion of knowledge of Kallikrein physiology. The first kallikrein protein was identified in the 1930s as the most abundant protease in the pancreas. It was subsequently named kallikrein for the Greek word for pancreas (kallikreas) (18). Three classical human tissue kallikreins (kallikrein 1, 2 and 3) were identified and their genes were localized in the 1980s. The 1990s and 2000s resulted in the discovery of new kallikreins, resulting in knowledge of tissue kallikreins 1-15 as well as a pseudogene ψKLK1 (19, 20). In addition to human kallikreins have been identified in a variety of animal species including monkeys, orangutans, mice, rats, dogs, cats, horses, pigs, cows and elephants (21).

Human kallikreins can also been divided into a plasma kallikrein and several tissue kallikreins based on their molecular biology and function. Tissue kallikreins are encoded on chromosome 19 and are a group of contiguously encoded proteases. Unlike the tissue kallikreins, the plasma kallikrein is encoded by a single gene (*KLKB1*) located at chromosome 4q35and has no similarities to the tissue kallikrein genes. Plasma kallikrein is secreted by hepatocytes as a plasma pre-kallikrein, an inactive precursor and is activated to its mature form by factor XII in the coagulation cascade (22). Activation of plasma kallikrein results in the release of bradykinin, with function in the coagulation cascade, regulation of vascular tone and inflammation (22).

3.1 Human Tissue Kallikreins

Human tissue kallikreins (KLKs) are a group of 15 single chain serine proteases with genes (*KLK1-KLK15*) encoded at chromosome 19q13.4 (23). The 15 KLKs (numbered KLK 1- 15) constitute the largest group of contiguously encoded proteases in the human genome (23). KLK1, KLK2 and KLK3 (prostate specific antigen or PSA) are known as the classical kallikreins. KLK2 and 3 are the most closely related, sharing 80% similarity at the amino acid and DNA level (21). Tissue kallikrein peptides are represented with the short-form ‘KLK’ followed by their number (1-15), while their

respective genes are noted with the abbreviation '*KLK*'. KLK 2-15 are officially named "kallikrein related peptidases" while the *KLK1* gene is known as Kallikrein 1 (24).

Tissue and Cellular Localization of Kallikreins

KLKs are expressed in a wide variety of human tissue types in normal and pathologic states. Research has identified certain tissue types with high and low levels of expression of specific *KLK*s in various tissues, as well as the presence of secreted KLKs in biologic fluid such as seminal plasma, serum and milk (21). In addition to identification of *KLK* mRNA in many different tissue types, multiple *KLK*s are often expressed in single tissue types, such as the salivary glands, which express nearly all *KLK*s (25). Multiple *KLK* mRNAs are also identified in the skin (*KLK1, KLK4-11, KLK13-14*), prostate (*KLK2-4, KLK11, KLK15*), breast (*KLK5-6, KLK10, KLK13*), pancreas (*KLK1, KLK6-13*) and central nervous system (*KLK6-9, KLK14*) (21). Alternative *KLK* transcripts have been linked to specific tissue types; an example of this includes splice variants of *KLK2* and *KLK3* which are identified exclusively in prostatic epithelium (21). Localization of KLK has been studied largely utilizing immunohistochemical analysis. KLK protein may be secreted, or sequestered to various regions of the cell. KLK 5 and 7 are localized to the granular cell layer of normal skin, KLK3, 4, 6, 7, 9, 10, 11, 13 and 14 are primarily found in the cytoplasm of glandular epithelium (21).

Regulation of Kallikreins

KLK regulation occurs at multiple levels including at the post-translational, transcriptional and post-transcriptional levels. Transcriptional control of *KLKs* occurs through many avenues. In endocrine tissue, all *KLKs* appear to be controlled by steroid hormones; steroid hormones and vitamin D receptors alter transcription of *KLK* genes in other tissues as well (26). Steroid control of *KLKs* can occur via indirect and direct mechanisms. Direct steroid transcriptional control occurs through the binding of steroid hormone-receptor complexes to promoter/enhancer regions at 'hormone response elements', which function by recruiting necessary cofactors to enhance transcriptional activity. Indirect steroid transcriptional control functions through trans-acting

transcription factors (21). Specific examples of steroid control include the upregulation of *KLK2* and *KLK3* in response to progestin and androgen action in breast and prostate cancer cell lines (21, 27). Androgen response elements have been identified in the promoter region of *KLK2 & 3*. *KLK4* is upregulated by estrogens in endometrial cancer cell lines, and also upregulated by androgens in prostate cancer cell lines (21). It is postulated that relative levels of activators/repressors contribute to the potential for up or down regulation of transcriptional activity in each *KLK* (21, 27). Additional methods of transcriptional control include epigenetic modifications including methylation and histone modification (23). Methylation of an exon resulting in downregulation of *KLK10* has been observed in acute lymphoblastic leukemia and breast cancer (21, 26).

Tissue kallikreins are translated as inactive precursors or zymogens, which have pro-peptides physically blocking their active site. To be activated, the signal peptide, a 16-30 amino acid segment at the NH2 terminus is cleaved in the secretory pathway resulting in the pro-KLK. Final activation often involves cleavage of 4-9 amino acid pro-peptides resulting in the active peptide (21, 26). KLKs can be activated by trypsin like serine proteases, via endopeptidases or autocatalytic activation (21, 26).

Glycosylation of the KLK proteins is believed to be important for regulation of proper KLK expression and function. KLKs and pro-KLK proteins have been identified to be involved in kinin mediated signaling cascades such that variably expressed kallikrein proteins can activate or inhibit expression of downstream kallikrein proteins (20). As a result, multiple KLKs can be involved in one, or multiple signaling cascades ultimately controlling biologic function. The roles of multiple KLKs acting on one another and their respective zymogens has been described as the 'KLK activome', and illustrates the complexity of the KLK family of proteins (26).

When activated, KLKs can be inactivated by a multitude of autolytic and enzymatic methods (23). Kallistatin is an example of an endogenous inhibitor responsible for the inactivation of KLK1 (26). KLK 6, 7, 13 and 14 have been shown to be capable of autoinactivation in vitro (21). Serine protease inhibitors, known as serpins are implicated in KLK inactivation via the 'inhibitory pathway'. In this pathway, serpins

such as protein C inhibitor (KLK2), α2-macroglobulin (KLK3, 5), α1-antichymotrypsin (KLK3), antithrombin III (KLK6) and α2-antiplasmin (KLK5, 6, 13) produce complexes that deform and irreversibly inactivate the various KLK proteins (21, 26). Local physiologic conditions, including pH and the relative abundance of zinc can contribute to reversible inhibition of KLKs (26). Plasmin has been identified to internally cleave KLK11 resulting in its inactivation (26).

Through expression in these signaling cascades, as well as modification of *KLKs* at the transcriptional, translational and post-translational levels of action, *KLKs* and their proteins are capable of contributing to physiologic function and/or disease progression (20).

Physiologic Roles of Kallikreins:

Kallikreins have been identified in a variety of tissues at the mRNA and protein levels (21). Some tissues (such as salivary glands, skin and the central nervous system) express multiple kallikrein proteins. As a result, KLKs have been investigated as biomarkers, characterized for their relative abundance in disease states and investigated for their roles in normal physiology. In general, five distinct methods of KLK mediated cell signaling have been identified (28). These include the modulation of cell signaling via the activation, generation or inactivation of peptide agonists from precursor proteins (including hormones), the activation or inactivation of growth factor receptors (i.e. tyrosine kinase growth factor receptors), cell signaling via extracellular matrix proteins and integrins, the release of growth factor receptor agonists bound to cell membranes, as well as the activation of proteinase activated receptors (i.e. G-protein coupled proteinase activated receptors) (28). As a result of their ability to function in many different pathways, KLKs can function in autocrine, paracrine and endocrine signaling utilizing the proteinase activated receptor method of signaling (29). Specific examples of various KLKs with resultant physiologic function will be described below.

KLK 1 is involved in the kinin-signaling cascade, cleaving low molecular weight kininogen, releasing lysyl-bradykinin. The resultant binding of lysyl-bradykinin to target receptors plays a role in modulating a wide variety of physiologic processes including

blood pressure regulation, smooth muscle contraction, vascular permeability, inflammatory cascades, neutrophil chemotaxis and pain induction (21, 23). These kinin mediated processes are especially vital in pregnancy, when the KLK 1 mediated cascades are involved in the control of placental blood flow, cellular proliferation and trophoblast invasion (21). KLK 1 has been implicated in additional physiologic processes unrelated to the kinin cascade. These include the activation of proinsulin to insulin and the processing of growth factors (21). KLK 1 has been identified to have a protective effect against lupus and antibody induced nephritis (30) and may play a role in calcium homeostatic regulation (26). KLK 2 and 3, the remaining classical kallikrein proteins also have well-established physiologic roles. KLK3/PSA is the most widely known kallikrein as a result of utilization in monitoring prostate cancer (26). Seminal clot liquefaction and subsequent sperm motility is partly controlled by KLK 2 and 3 through the action of these kallikrein proteins hydrolyzing seminogelin I, II and fibronectin after ejaculation (31, 32). KLK 3 functions more efficiently in the cleavage of the aforementioned substrates. Activation of KLK 3 is dependent on a reduction in the high concentration of zinc found in seminal fluid, which occurs through binding of zinc to seminogelin I and II (26). KLK 2 may also contribute to the activation of KLK 3 in prostatic tissue (33). Additional physiologic KLK 3 substrates include lysozymes and growth factor binding proteins (21).

KLK 4 has been studied for its role in the formation of normal tooth enamel, a process known as amelogenesis where it functions to degrade enamel proteins (34). Research in porcine and mouse models suggests the role of KLK 4 in enamel matrix processing during amelogenesis (21, 34). KLK 4 has been found to be secreted and expressed by murine ameloblasts (34). A mutation in the *KLK4* gene has been identified in some patients suffering from one form of amelogenesis imperfecta, a disorder of abnormal enamel formation (23, 35). Primary teeth from humans with KLK 4 mutation exhibit opaque enamel deposition in comparison to control enamel. KLK 4 deficiency results enamel with excess protein, abnormal crystal growth, but normal crystal orientation, prism orientation and enamel thickness (36). Furthermore, in *KLK*4 null mice and humans with mutations in the KLK4 gene, defects are noted in enamel, but not elsewhere in the body (34). Using quantitative PCR, *KLK 4* was also identified in

prostate, adrenal, thyroid and salivary gland tissue (34). In these tissues, the physiologic role of KLK 4 has not been determined.

KLK 5, 7 and 14 have been identified in the granular cell layer of the epidermis and are found to degrade epithelial desmosomes (21, 26). KLK 5 possesses the ability to auto-activate, and is implicated as a common activator (along with matrix metalloproteases and urokinase-type plasminogen activator) in a number of KLK signaling cascades; it has been demonstrated to activate pro-KLK 2, 3, 6, 7, 11, 12 and 14 (21, 26). Through the activation of KLK 7 and 14 by KLK 5, epithelial adhesion proteins desmoglein, corneodesmin and desmocollin (located in the stratum corneum), can be broken down to facilitate normal epithelial turnover. KLK 14 has been implicated in a feedback loop in this epithelial turnover, and as a result, is involved in the skin desquamation cascade through two effects of the protein (21, 26). KLK 5 and 7 also contribute to the antimicrobial property of skin through their activation of human cathelicidin precursor protein CAP18, activating cathelicidin to its mature form, which possesses these antimicrobial properties (23, 26).

KLKs have been identified in other locations of a variety of other tissue types. KLK 5, 6, 7, 8, 10, 11, 12 and 13 are present in cervicovaginal fluid in very high concentrations. It is postulated that they may be involved in the desquamation of vaginal epithelium, and possibly may have roles in processing of antimicrobial peptides found in vaginal fluid (26). Immunohistochemical investigation has demonstrated the presence of KLK 3, 4, 6, 7, 9, 10, 11, 13 & 14 in the cytoplasm of glandular epithelium (21). In addition to KLK 5, KLK 2, 6 and 13 are also capable of autoactivation (21). In vitro studies have identified the potential of KLK 3, 5, 6, 13 and 14 to cleave extracellular matrix proteins, implicating them in tissue remodeling (21). KLK 6 is produced by oligodendrocytes, and is involved in normal remyelination and the maintenance of normal neuronal myelination through the cleavage of myelin basic protein (29).

Additional physiologic processes with KLK involvement include smooth muscle contraction, neutrophil chemotaxis, inflammation, angiogenesis and pain induction (23).

Kallikreins in Pathologic States:

Overexpression of some KLKs is found in malignant and non-cancerous disease states. Studies have focused on the abundance of KLK in serum, identification of KLK in tissue via immunohistochemistry and identification of relative levels of *KLK* mRNA. The mechanisms resulting in altered *KLK* expression have not been well described for many disease states. In addition, the exact pathway by which altered *KLK* expression or altered KLK protein levels contribute to disease states is not always clear. Despite this, some mechanisms of carcinogenesis including tumour growth, angiogenesis and invasion have been correlated with altered KLK expression (21). Synergistic effects of kallikrein proteolytic cascades where there is increased co-expression of multiple KLKs may result in increased tumour growth compared with expression of unique KLKs alone (23). Additionally, *KLK* overexpression has been identified as a prognostic marker in malignancy (21). The following paragraphs will address some of the known changes in KLK biology in pathologic states.

Kallikrein proteins have been identified to inhibit or promote carcinogenesis depending on their expression. KLK 2, 3, 5, 6, 13 and 14 are known to directly catalyze the breakdown of extracellular matrix (ECM) proteins, subsequently contributing to direct tumour invasion, as well as potential metastasis (21). Indirectly, KLK 2 and 4 can promote invasion and metastasis through the activation of urokinase type plasminogen activator, which activates plasminogen to degrade ECM proteins (37, 38). In the prostate KLK 3 also indirectly promotes tumour invasion and metastasis through the activation of insulin like growth factor and activation of transforming growth factor beta (TGF-β). TGF-β promotes invasion through cell detachment, and insulin like growth factor is mitogenic for prostatic stromal and epithelial cells (39). Through its kinin activity, KLK 1 promotes an increase in vascular permeability, regulates angiogenesis, and supports proliferation of tumour cells (21). Wolf et al (40) identified a synthetic inhibitor of KLK1 that suppressed the invasive potential of breast cancer cell lines in vitro.

KLK 4, 5, 6, 7 and 15 mRNA expression and KLK 5, 6, 7, 10 protein expression has been identified as a negative prognostic marker in ovarian cancer (21). In contrast,

KLK 8, 9, 11, 14 expression and KLK 13 expression have been identified as favourable prognostic markers in ovarian cancer (21). Splice variants or alternative transcripts of *KLKs* have been identified in various forms of malignancy/disease. In ovarian cancer, overexpression of *KLK* 5 and *KLK* 7 splice variants has been observed compared to normal ovarian tissue (41). In addition, a unique *KLK 8* variant was identified in ovarian cancer tissue, and not identified in normal ovarian cells (42).

Observations of *KLK* expression in breast cancer provide other interesting observations about KLK biology. *KLK* 6 is upregulated in primary breast tumours, but down regulated at metastatic breast cancer sites (43). Additionally, levels of KLK 5 and 14 in serum of breast cancer patients were elevated, while the *KLK* 5 and 14 mRNA expression was reduced in tissue (44, 45). *KLK* 3, 10, 12, and 13 mRNA are also downregulated in breast cancer cell lines or tissues (21). *KLK 10* is considered a tumour suppressor gene and when injected into breast cancer cell lines, tumour formation in nude mice was decreased (21). Downregulation of *KLK* 10 in breast, ovarian, prostate and acute lymphoblastic leukemia is attributed to hypermethylation of exon 3 (46, 47). Epigenetic modifications may also be responsible for other alterations in KLK expression in disease states.

KLK 3 levels have been identified to be elevated in serum prostate cancer patients, while decreased in tissue (21). It is postulated that the malignancy/carcinogenesis, with associated angiogenesis and/or the loss of normal glandular architecture contribute to an increased release of various KLKs from tissue, resulting in an increased serum level. In prostate cancer bony metastasis may be mediated by KLK 4; in vitro studies demonstrate that KLK 4 improves prostate cancer cell migration against osteoblast secreted factors (48). mRNA quantification reveals that *KLK* 2, 3, 5, 6, 10 and 13 are down-regulated, while *KLK* 11, 14 and 15 are overexpressed in prostate cancer (21). In addition to its role as a serine protease, in prostate cancer cell KLK3 produces reactive oxygen species (26).

Other malignancies identified to have aberrant *KLK* gene expression include lung adenocarcinoma where *KLK* 11 is overexpressed in tumour of neuroendocrine origin,

pancreatic adenocarcinoma where overexpression of *KLK 10* is observed and acute lymphoblastic leukemia where *KLK* 10 is downregulated (21). Utilizing immunohistochemical evaluation, KLK 10 levels were found to be decreased in pleomorphic adenoma specimens compared to normal salivary gland tissue (49). KLK 8 was found in normal and pathologic salivary gland tissue, and increased levels of KLK 8 were identified in mucoepidermoid carcinoma as well as adenocarcinoma NOS (50). Normal salivary gland tissues, as well as multiple benign and malignant tumour types were identified to have KLK 14 expression using immunohistochemical analysis. Pleomorphic adenoma and adenoid cystic carcinoma tissues were identified to have higher levels of KLK 14 relative to normal salivary gland control, as well as other tumours investigated (51).

Benign disease states involving the neurologic, dermatologic and respiratory systems are correlated with altered KLK expression. KLK 6 is found secreted by mouse inflammatory cells in the central nervous system where it is understood to cleave basic myelin proteins (29). KLK 1 and 6 are found to cleave alpha-synuclein, a large component of the insoluble protein found in Lewy bodies, part of the underlying pathology for Parkinson's disease and Lewy body dementia (29). Uncontrolled activation of KLK 5, 7 & 14 is identified to be a cause of over desquamation of skin in peeling skin syndrome (23). KLK 7 may be involved in additional skin pathoses including psoriasis, and inflammatory reactions due to the involvement of KLK 7 activating pro-inflammatory interleukins (21). Netherton syndrome is a genetic disorder characterized by a mutation in the *SPINK5* gene which is responsible for coding for the serine protease inhibitor kazal type 5. The aforementioned mutation results in patients suffering from the uninhibited proinflammatory effects of KLK 5 and 7 which signal via proteinase activated receptors to contribute to the dermatologic abnormalities observed in this syndrome (28). KLK 1 is the principle kinin generating enzyme of the human airways and is implicated in bronchoconstriction, production of mucous and mediation of vascular permeability (29, 52).

Table 3.1 – Known or Proposed Roles as well as Association of KLK Protein and *KLK* genes in Physiologic and Pathologic States

Known or Proposed Role or Association	Kallikrein Protein or Gene (*KLK* in Italic Font) Associated	Refererence
Regulation of Blood Pressure, Smooth Muscle Contraction, Inflammatory Cascades	KLK 1	21, 23, 26, 30
Regulation of angiogenesis, vascular permeability, support of tumour cell proliferation	KLK 1	21,23, 26
Semen Liquefaction	KLK 2, 3 6, 7, 8, 14	26, 31, 32, 33
Skin Desquamation/Epithelial Turnover	KLK 5, 6, 7, 8, 10, 13, 14	21, 26
Desquamation of Vaginal Epithelium, Antimicrobial Effect of Vaginal Fluid	KLK 5, 6, 7, 8, 10, 11, 12, 13	26
Antimicrobial Activity of Skin	KLK 5, 7	21, 23, 26
Extracellular Matrix Remodelling	KLK 3, 5, 6, 13, 14	21
Tumour Invasion via Direct Extracellular Matrix Degradation	KLK 2, 3, 5, 6, 13	21
Activation of Urokinase Type Plasminogen Activator (results in Tumour Invasion)	KLK 2, 4	37, 38
Tumour Invasion via TGF-Beta and IGF Activation	KLK 3	39
Amelogenesis	KLK 4	21, 34, 35, 36
Pro-Hormone Activation (i.e. of Insulin, Growth Hormone, Glucagon, Somatostatin, Pancreatic Peptides)	KLK 1, 3, 6, 10, 13	21
Myelination & Synaptogenesis	KLK 6, 8	29
KLK Autoactivation	KLK 2, 5, 6, 13	21
Favourable Prognostic Marker in Ovarian Cancer	*KLK* 8, 9, 11, 14 KLK 13	21

Negative Prognostic Marker in Ovarian Cancer	*KLK* 4, 5, 6, 7, 15 KLK 5, 6, 7, 10	21
Breast Cancer	Elevated serum KLK 5 & 14 Decreased *KLK* 5, 14 expression in breast cancer tissue Downregulated *KLK* 3, 10, 12, 13	21, 44, 45
Tumour Suppressor Gene (in Breast Cancer cell lines)	*KLK* 10	21
Prostate Cancer	Improved bony metastasis – KLK 4 Down regulated *KLK* 2, 3, 5, 6, 10, 13 Overexpressed *KLK* 11, 14, 15	21, 26, 48
Lung Adenocarcinoma	Overexpression *KLK* 11	21
Pancreatic Adenocarcinoma	Overexpressed *KLK* 10	21
Mucoepidermoid Carcinoma, Adenocarcinoma NOS	Increased KLK 8	50
Pleomorphic Adenoma, Adenoid Cystic Carcinoma	Increased expression KLK 14	51
Normal Salivary Gland	KLK 8, 14	50
Psoriasis	KLK 7	21
Bronchoconstriction	KLK 1	29, 52
Peeling Skin Syndrome	KLK 5, 7, 14	23
Parkinson's Disease	KLK 1, 6	29

Kallikreins as Biomarkers:

Biomarkers are useful in medicine for screening, diagnosis, prognosis, staging disease and following disease after treatment. Kallikrein proteins have been used as biomarkers for 3 decades, originally with the identification of serum KLK 3 (Prostate Specific Antigen) and its association with prostate cancer. Due to their genetic similarity, and relative physiologic abundance, KLK level and *KLK* expression have been investigated in a number of additional disease states for their roles as serum or tissue biomarkers (18).

Limitation of the specificity of the serum PSA has resulted in the utilization of specific logarithmic regression models comparing KLK 2 to free PSA as well as free PSA to total PSA in order to improve the diagnostic accuracy of biomarkers for prostate cancer compared to benign prostatic hypertrophy (18). Additionally, expression of *KLK 5, 11, 14* and *15* may correlate with Gleason biopsy grade, allowing the gene expression level to act as a prognostic marker in prostatic carcinoma (18, 20). Utilization of KLKs with additional serum markers has also been found to increase the diagnostic accuracy in detection of prostate cancer. Utilizing an analysis involving free PSA, KLK 11, migration inhibitory factor and macrophage inhibitor cytokine 1 improves the ability to identify prostate cancer from benign prostatic hypertrophy (53). In testicular cancer, *KLK 5* expression has been correlated with improved outcomes and therefore may represent a positive prognostic marker (20).

Identifying certain KLKs in ovarian and breast cancer aids in establishing the diagnosis, as well as with prognosis (23). KLK 6 is associated with late stage, high grade ovarian cancer. In addition, KLK 4, 5, 6, 7, 10 and 15 may correlate with poor prognosis in ovarian malignancy (18, 20, 21, 23). KLK 8 and 14 may represent favourable prognostic markers in ovarian cancer. Expression of *KLK* 4, 5, 6, 7 and 15 mRNA has been identified as a negative prognostic marker, and *KLK 4* may predict resistance to paclitaxel chemotherapy, and may be therefore useful in guiding treatment (54). In contrast, *KLK* 8, 9, 11 and 14 expression and KLK 13 expression have been identified as favourable prognostic markers in ovarian cancer (21). To aid with pathologic diagnosis

of primary ovarian malignancy versus metastatic disease, the presence of KLK 8, 10, 13 and Cancer Antigen 125 (CA 125) have been found to be superior to CA 125 alone (18). Breast cancer investigation has revealed that increased expression of KLK 3, 9, 13 and 15 may indicate favourable prognosis while expression of KLK 5, 7 and 14 represent poorer prognosis (18, 20). While still under investigation it is possible that expression of KLK 3 and 10 may predict response to tamoxifen therapy (20).

Additional malignancies where KLK levels or *KLK* expression may be of clinical benefit include renal cell carcinoma, where *KLK 6* expression is negatively correlated with disease free survival (18). KLK 6 has been identified to be upregulated in pancreatic, esophageal and gastric cancer; in these malignancies it carries with it an unfavourable prognosis (18, 20). KLK 7, 8, 12, 13 and 14 have been identified as potential serum markers for lung cancer. In neuroendocrine type lung cancer, upregulation of *KLK11* has been linked with worse outcomes (55).

Evaluation of epigenetic modifications such as methylation of kallikrein genes may also prove beneficial in the evaluation of pathologic states. Methylation of *KLK10* has been associated with a poorer prognosis and increased chance of relapse in acute lymphoblastic leukemia (18).

Following their roles as biomarkers, the wide array of physiologic roles displayed by KLKs in multiple tissue types has led to interest in targeting KLK expression/activity for therapeutic intervention.

Possible Therapeutic Approaches Utilizing Kallikreins:

In addition to their role as biomarkers, kallikrein proteins and *KLK* expression are potential therapeutic targets to alter disease progression. Inactivation of KLKs may occur through cleavage (including auto-inactivation) and via serine protease inhibitors (known as serpins). A single serpin may inhibit multiple proteases (KLKs), and each protease can potentially be targeted by multiple inhibitors. Serpins work through ‘inhibitory’ and ‘substrate’ pathways. The inhibitory pathway results in formation of a complex that irreversibly deforms the protease. Substrate inactivation is reversible, and

occurs through preventing interaction of the proteases with potential substrates as a result of steric hindrance (21). KLK inhibitors have been identified for KLK 1, 3 and 7 but the biologic significance of these inhibitors is not yet fully understood (29). Therapeutic approaches may take advantage of tissue specific expression of KLKs. Development of pro-drugs with KLK specific peptide sequences could allow clinicians to minimize systemic exposure of the drug, and maximize the local tissue exposure to areas of high expression of the desired KLK (29). Development of specific KLK aptamers may provide another option for therapeutic intervention of KLK mediated disease processes.

Proteinase activated receptors are known to be involved in signaling for a number of dermatologic disorders (28) and may provide a potential avenue for therapeutic intervention. Modification of the KLK – proteinase activated receptor pathway could potentially help maintain skin homeostasis in disorders such as Netherton syndrome, peeling skin syndrome type B, psoriasis, rosacea and atopic dermatitis (28, 29).

Neuropathologies are another broad category of diseases that could benefit from KLK specific interventions. KLK8 deficient mice have been identified not to show anxiety in response to stress provoking conditions (29). Intranasal administration of recombinant KLK 1 reduces the rate of degeneration of the substantia nigra in a mouse model of Parkinson's disease (29). Targeting KLK 6, with a resultant blockage of KLK 6 activity in rodents delayed the onset and reduced the severity of the rodent model of multiple sclerosis (29). Monoclonal antibodies are a potential method of delivering some of these anti KLK 6 actions; in a mouse model, treatment with anti KLK6 neutralizing antibodies resulted in a decrease in the loss of basic myelin protein and a delay in the progression of the demyelination (56). Monoclonal antibodies developed to act as a competitive inhibitor to KLK 1 have also been efficacious in a sheep model at reducing bronchoconstriction and airway hyper-responsiveness; a potential therapeutic approach for reactive airway disease (52).

Chapter 4

4 Hypothesis and Rationale

4.1 Hypothesis

We hypothesize that human kallikrein proteins 3, 4, 5, 9 and 11 are expressed in the following odontogenic tumours and cysts; ameloblastoma, KOT, dentigerous cyst and lateral periodontal cyst. In this study we expect to find altered levels of kallikrein proteins in the odontogenic cysts and tumours selected compared to our control tissues, an odontogenic hamartoma (odontoma) and non-odontogenic maxillofacial cyst (nasopalatine duct cyst). Additionally, we hypothesize that multiple *KLK* genes will be expressed in ameloblastoma tissue samples and that the expression of *KLK* genes will correlate with KLKs identified utilizing immunostaining.

4.2 Rationale

Odontogenic, non-inflammatory maxillofacial cysts and tumours vary greatly in their ability to grow and cause local tissue destruction. Despite their common embryologic origin, the biologic mechanisms responsible for this diverse array of clinical behaviour are largely unknown. While the natural history of these neoplasms is known, treatment strategies remain controversial for the KOT and ameloblastoma. Management strategies for these cysts and tumours depend on the histologic diagnosis made from biopsy specimens. Unfortunately, even with accurate tissue diagnosis and appropriate surgical management, these tumours have relatively high recurrence rates (6, 7). While this may be related to surgical technique, it may also be due to intrinsic tumour biology. Additionally, there are no proven medical management strategies for these tumours, and only some of these lesions lend themselves to minimally invasive surgical techniques such as marsupialization. Due to their location in the facial skeleton, tumours causing significant local tissue destruction can be quite disfiguring, leading to altered physical appearance and oral function following their resection and surgical reconstruction(7). As a result, a greater understanding of the molecular mechanisms responsible for the varied clinical behaviour of these tumours would help improve our understanding of their

biology. Furthermore, creating a profile of the proteins present or genes expressed in each tumour or cyst may provide pathologists with a tool to assist in histopathologic diagnosis. The ultimate goal of establishing biomarkers for these odontogenic cysts and tumours would ideally be to provide clinicians an avenue to diagnose these neoplasms without tissue biopsy or to allow analysis of the intrinsic features of each patient's tumour to help predict recurrence, therefore guiding treatment. With an improved understanding of the tumour biology, it may be possible to develop strategies for medical or minimally invasive management of these neoplasms.

Kallikrein proteins have been evaluated as biomarkers, investigated for their role in molecular signaling cascades in multiple tissue types, and have been implicated in tumour growth, angiogenesis, invasion and metastasis. Our lab has experience in the investigation of head and neck neoplasms of salivary and odontogenic origin for the presence and relative abundance of kallikrein proteins (25, 49, 50).

4.3 Aims and Objectives

This study aims to provide additional information on the pathogenesis and increase the spectrum of diagnostic markers available for evaluation of odontogenic maxillofacial neoplasms and cysts. Utilizing immunohistochemistry, we will investigate the presence and relative abundance of KLK 3, 4, 5, 9 and 11 in odontogenic epithelium including basal layer cells, clear cells, preameloblasts, stellate reticulum-like cells, squamous cells and parakeratin in tissue from the following odontogenic tumours and cysts; ameloblastoma, KOT, dentigerous cyst, lateral periodontal cyst, odontogenic hamartoma (odontoma) and non-odontogenic maxillofacial cyst (nasopalatine duct cyst).

Reverse transcription – polymerization chain reaction (RT-PCR) will be used to evaluate for the presence of mRNA for the 15 human kallikrein proteins in ameloblastoma tissue. We will then compare the mRNA expression with the KLK protein identified utilizing immunohistochemistry (where possible) based on data from our laboratory. Using this data, we will evaluate whether altered expression and/or unique KLK profiles help to predict behaviour of these lesions.

Chapter 5

5 Materials and Methods

5.1 Materials for Immunostaining

Tissue samples for the tumours, cysts and control tissues were obtained from the division of Oral Pathology, Schulich School of Medicine and Dentistry, Western University and London Health Sciences Centre. Multiple hematoxylin and eosin stained slides of each tissue type were assessed by the graduate student (KC) as well as two experienced oral and maxillofacial pathologists to determine if sufficient lesional tissue was present for immunostaining purposes. Ultimately, sixty (n=60) formalin fixed paraffin embedded specimens were selected for investigation from the archives of the Division of Oral Pathology. These included lateral periodontal cysts (n=9), dentigerous cysts (n=10), KOTs (n=11) and ameloblastomas (n=11) as well as nasopalatine duct cysts (n=9, non-odontogenic cystic control) and odontomas (n=10, hamartoma as neoplasm control). Patient demographics including age, sex, and site of lesion are noted in tables 5.1 – 5.6.

Table 5.1 – Lateral Periodontal Cyst Tissue Demographic Information

Case Number	Age	Sex	Site
1	84	Female	Anterior mandible
2	61	Female	Anterior mandible
3	75	Male	Left maxilla premolar area.
4	79	Female	11,21 area
5	70	Male	Left mandible
6	62	Female	Left mandible 34 area
7	58	Male	Right mandible 43/44
8	59	Female	Left mandible
9	80	Female	44/45 area

Table 5.2 – Dentigerous Cyst Tissue Demographic Information

Case Number	Age	Sex	Site
1	48	Male	impacted 48
2	40	Female	Impacted 38
3	40	Female	impacted 48
4	43	Male	impacted 48
5	55	Female	impacted 48
6	26	Female	Impacted 38
7	45	Male	Impacted 38
8	49	Male	impacted 48
9	50	Male	impacted 48
10	32	Male	impacted 38

Table 5.3 – Keratocystic Odontogenic Tumour Tissue Demographic Information

Case Number	Age	Sex	Site
1	34	Male	Right mandible 48 area
2	32	Female	Left posterior mandible.
3	71	Male	Mandible 33 and 35 area
4	19	Female	Left ramus
5	64	Male	Right mandible 46 area
6	44	Male	Right mandible
7	46	Male	Mucosa between 27 and 24
8	57	Male	Left body and ramus manidble
9	67	Male	Maxilla 24 area
10	20	Male	Left mandible 38 area
11	52	Male	Left maxilla 24/25

Table 5.4 – Ameloblastoma Tissue Demographic Information

Case Number	Age	Sex	Site
1	82	Female	Lingual to 31 to 42.
2	74	Male	Left anterior mandible
3	35	Female	Left posterior mandible
4	55	Male	Left ascending ramus
5	51	Female	Left mandible
6	75	Female	Maxilla/sinus
7	15	Female	38 area
8	75	Female	Right maxillary sinus
9	52	Male	Right mandible 46 area
10	57	Male	32/33 area
11	26	Female	35 area

Table 5.5 – Nasopalatine Duct Cyst Tissue Demographic Information

Case Number	Age	Sex	Site
1	81	Male	Right maxilla 11 area
2	32	Female	11,21 area
3	78	Male	Incisive canal
4	53	Male	Anterior maxilla
5	12	Male	Anterior maxilla 11/21
6	67	Female	Anterior maxilla
7	59	Male	Right anterior maxilla
8	38	Female	Anterior maxilla
9	35	Male	Maxillary midline

Table 5.6 – Odontoma Tissue Demographic Information

Case Number	Age	Sex	Site
COMPLEX ODONTOMA			
1	29	Female	Maxilla 22/23 area
2	19	Female	Left posterior maxilla
3	18	Female	Mandible 38 area
4	18	Male	Maxilla 28 area
5	10	Female	Maxilla 16 area
6	7	Female	Right mandible 85 area
7	13	Male	Left mandible apical to 73
8	17	Female	Left maxillary tuberosity
COMPOUND ODONTOMA			
9	17	Male	Left mandible 32/33 area.
10	11	Female	Right anterior maxilla

Table 5.7 – Patient Demographics for Study Lesion Population

Lesion Type	Number of Specimen	Mean Age (years)	Gender (Male:Female)
Lateral Periodontal Cyst	9	69.8	3:6
Dentigerous Cyst	10	42.8	6:4
Keratocystic Odontogenic Tumour	11	46.0	9:2
Ameloblastoma	11	54.3	4:7
Nasopalatine Duct Cyst	9	50.6	6:3
Odontoma	10	15.9	3:7

Summary data outlining number of specimens investigated, mean age, and gender distribution are indicated in table 5.7

Diagnosis of the lesion was based on the World Health Organization classification of tumours (57). Evaluation of 10-11 lesions per tissue type was planned, but for nasopalatine duct cysts and lateral periodontal cysts there was insufficient tissue in some cases.

Positive staining control tissue was obtained based on known expression of KLK 3, 4, 5, 9 and 11 in various human tissue types (Table 5.8) (18-21, 23, 26, 29, 34, 45, 48). Positive control tissues were used to determine optimal antibody concentration, and as a result, appropriate staining intensity utilizing graded concentrations of antibody and the staining method described in the following section of text (5.7). Antibody dilutions are outlined in table 5.8

Evaluation of KLK 3, 4, 5, 9 and 11 in the tumours, cysts and control tissues were performed utilizing a standard immunohistochemical staining technique.

5.2 Methods for Immunostaining

Parrafin embedded tissue samples were cut into 5 μm thick serial sections using a microtome (Microm HM 325;GMI Inc., Ramsey, MN) then transferred to a water bath at 45°C. After mounting these sections on positively charged glass slides, they were dried overnight in an incubator at 37°C. Following this, slides were treated with xylene followed by ethanol dilutions then water. A 3% hydrogen peroxide in methanol solution was then used, and rinsed with distilled water and phosphate buffered saline (PBS) on a shaker. Antigen retrieval was performed using a pH 6.0 citrate buffer in a de-cloaking chamber. Slides were then rinsed and blocked using 10% horse serum. Incubation was next performed with the primary antibody as described in table 5.8. Excess antibody was then removed using a PBS rinse, and slifes were incubated utilizing the ImmPRESS® kit's (ImmPRESS® Reagent Kit; Vector Laboratories, Burlingame, CA) anti-appropriate antibody horse-radish peroxidase micro polymer solution. Slides were rinsed and then stained with Diaminobenzidine (DAB) solution (Vector Laboratories, Burlingame, CA). Counterstaining was performed utilizing hematoxylin and slides were then dehydrated using ethanol and xylene in series. Slides were then mounted and cover slips were applied utilizing Cytoseal® (VWR, Mississauga, ON).

Table 5.8 - Antibody and Incubation Protocol for Immunostaining

Kallikrein Protein	Antibody Used	Antibody Titre (μL antibody/μL HS)	Positive Control Tissue	Incubation
KLK 3	Rabbit (polyclonal)	1/800	Prostate	Overnight at 4°C
KLK 4	Rabbit (polyclonal)	1/600	Prostate	Overnight at 4°C
KLK 5	Mouse (monoclonal)	1/800	Seborrheic Keratosis	1 hour at Room Temperature
KLK 9	Goat (polyclonal)	1/100 = 10 ug/mL	Prostate	Overnight at 4°C
KLK 11	Mouse (monoclonal)	1/200 = 5 ug/mL	Prostate	1 hour at Room Temperature

5.2.1 Evaluation of Immunostaining

Immunostained slides were evaluated under light microscopy utilizing a semi-quantitative analysis previously described (25, 51). Cells evaluated for staining were the odontogenic epithelium including basal layer cells, clear cells, preameloblasts, stellate reticulum-like cells, squamous cells and odontogenic parakeratin. Staining was evaluated by two oral and maxillofacial pathologists as well as the graduate student author. Staining intensity and proportion of cells stained were assigned non-parametric scores as illustrated in Table 5.9 and 5.10. The staining intensity score and proportion of cells stained scores were summed to yield an overall staining score (OSS) ranging from 0-8 for each slide of tissue evaluated. An OSS was also generated for non-study, control tissue used to assess the quality of immunostaining (i.e. prostate tissue control) for quality control purposes. The average KLK overall staining score was then generated for each tissue type and compared with all other tissue types within each KLK investigated.

Table 5.9 - Staining Intensity Scoring System

Score	Staining Intensity
0	None
1	Weak
2	Moderate
3	Strong

Table 5.10 - Percentage of Cells Stained Scoring System

Score	Percentage of Cells Stained
0	0%
1	<1%
2	1-10%
3	10-30%
4	30-60%
5	>60%

5.3 Materials for Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Formalin fixed, paraffin embedded ameloblastoma tumours were obtained from the archives of the division of Oral Pathology at the Schulich School of Medicine and Dentistry, Western University and London Health Sciences Centre and sectioned into 10 μm thick samples. The following lesional tissue sections were pooled; patient demographic information is included in table 5.10.

Table 5.11 - RNA Obtained for Each Ameloblastoma Tumour Specimen

Formalin Fixed Ameloblastoma Tissue Case Numner	Concentration RNA (at 1% RNA) (ug/mL)
1	0.276
2	0.208
3	0.101
4	0.039
5	0.129
6	0.120
7	0.406
8	0.071
9	Insufficient RNA
10	0.885

5.4 Methods for RT-PCR

Ten μm thick samples of each tumour were placed into 1.5 mL collection tubes. The tissues were then deparaffinized by adding xylene. The samples were mixed and centrifuged at 12000 xg for 2 minutes. Supernatant was discarded and the deparaffinization steps were repeated 3 more times. Samples were then passed through absolute ethanol and 70% ethanol, mixing and centrifuging (12000 xg for 2 minutes) for each step. Next, total RNA was isolated from these deparaffinized samples using Roche formalin fixed paraffin embedded RNA isolation kit (High Pure FFPE RNA Micro Kit; Roche Applied Sciences, 2011, Mannheim, Germany, Catalogue number: 04823125001). Briefly, tissue lysis buffer containing sodium dodecylsylphate was added to the tissue pellets. Samples were then centrifuged and digested with proteinase K for 3 hours. RNA was then isolated using High Pure RNA binding spin columns as recommended by the manufacturer.

Total RNA was measured using Qubit® Quant-iT™ RNA BR Assay Kit and Qubit® Fluorometer (Life Technologies). Nine out of ten ameloblastoma samples produced sufficient RNA for use in the synthesis of cDNA; the quantity of RNA is noted in Table 5.11. The 9 remaining ameloblastoma tissue samples were then pooled and cDNA was generated using 4 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). KLK primers are listed in Table 5.12, with specific catalogue number denoted for each primer. Reagents are noted in Table 5.13. RT-PCR reactions were performed in BioRad CFX Connect (Bio-Rad Laboratories) and comprised of 10 μL SYBR Advantage qPCR premix (Clontech Laboraories Inc., Mountain View, CA), 5.5 μL H_20, 2.5 μL cDNA, and 2 μL of primer for each KLK. Data was normalized to β-actin as a control/housekeeping gene. Reactions were completed as follows: 95^oC for 2 minutes (initial denaturation/activation), 95^oC for 5 seconds, 62^oC for 10 minutes for 40 cycles of amplification. Melting curve analysis was performed to determine specificity of the reactions.

Table 5.12 – PCR Gene Primers with Catalogue Information

Primer	Vendor	Catalogue Number
KLK 1	Qiagen	QT00020664
KLK 2	Qiagen	QT00088466
KLK 3	Qiagen	QT00027713
KLK 4	Qiagen	QT00495159
KLK 5	Qiagen	QT00010437
KLK 6	Qiagen	QT00013972
KLK 7	Qiagen	QT00028343
KLK 8	Qiagen	QT00017689
KLK 9	Qiagen	QT00057190
KLK 10	Qiagen	QT00039816
KLK 11	Qiagen	QT00011011
KLK 12	Qiagen	QT00067977
KLK 13	Qiagen	QT00029876
KLK 14	Qiagen	QT00039928
KLK 15	Qiagen	QT00035735
Beta Actin	Qiagen	QT01680476

Table 5.13 – PCR Reagents

Reagent	Vendor	Catalogue Number
ssoFast Evagreen PCR Kit	Bio-Rad	172-5201
iScript™ Reverse Transcription Supermix for RT-qPCR	Bio-Rad	1708841

5.5 Statistical Analysis

Mean overall staining scores and standard deviation of mean were calculated for all tissue types for each KLK of interest. Non-parametric statistical analysis was performed utilizing GraphPad software (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA). Dunn's multiple comparison test was used to evaluate for significance within each KLK type, and the Kruskall-Wallis test was utilized to examine for significance between overall staining scores of each tissue type within each set of KLK stained. Significance level for all tests was $p<0.05$.

The standard curve method of RT-PCR analysis was used to assess relative levels of KLK mRNA in the pooled ameloblastoma tissue. Since we wanted to confirm the presence of KLK transcripts (indicating expression of KLK) in the samples, we pooled the samples to yield PCR-quality cDNA. No statistical tests were performed due to the lack of comparison with other control tissue.

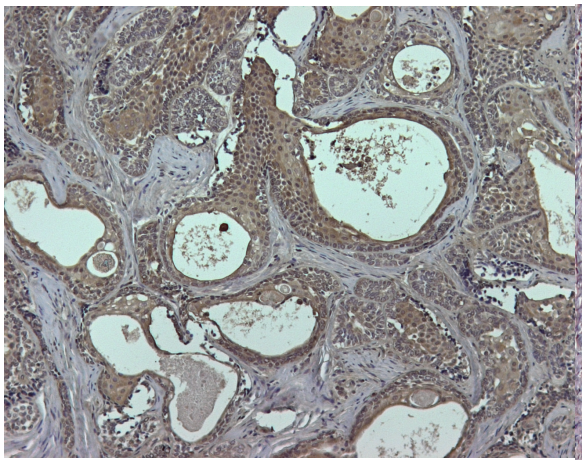
Chapter 6

6 Results

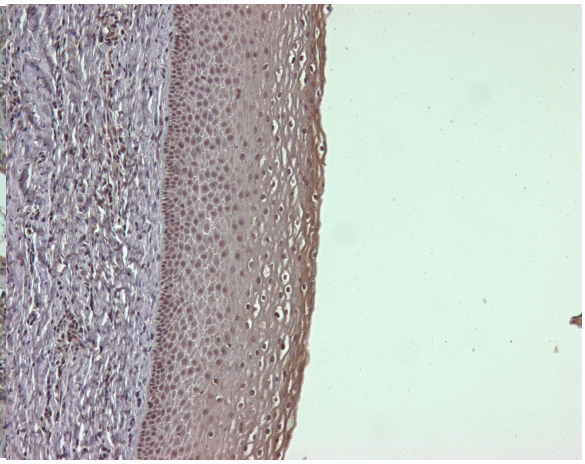
6.1 Immunostaining

Representative images were obtained for KLK 3, 4, 5, 9 and 11 staining in the control and study tissues. These images are displayed below (Figures 6.1 – 6.6) and were taken at 100X original magnification unless otherwise indicated on the figure legend where '50x' represents an image taken at 50X original magnification. Mean and median staining scores for all tissues stained are presented in tables 6.1 and 6.2. Figures 6.7 - 6.10 graphically depict the mean and median overall staining scores for KLK 3, 4, 5, 9 and 11 in the study tissues.

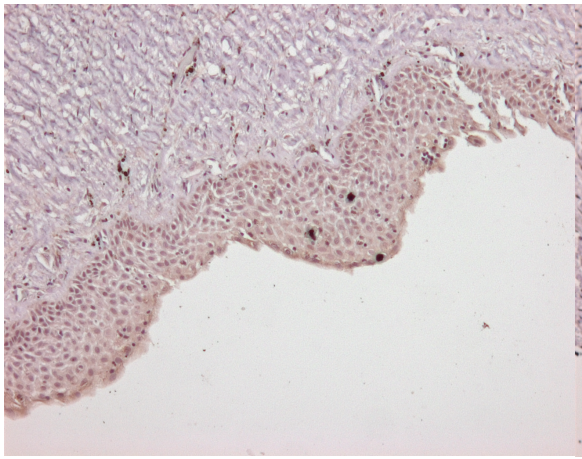
Figure 6.1 - KLK 3 Immunostaining (indicated by brown stain in tissues)



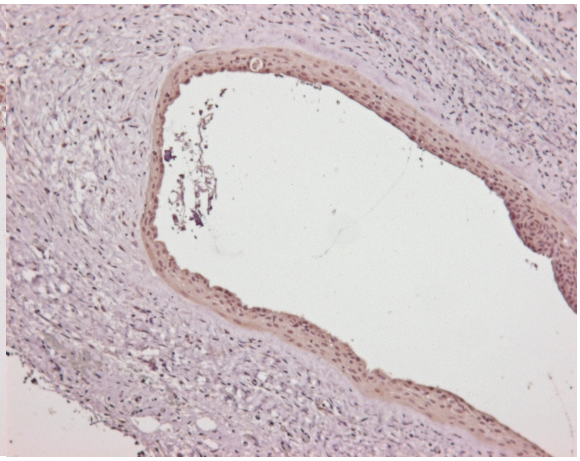
Ameloblastoma



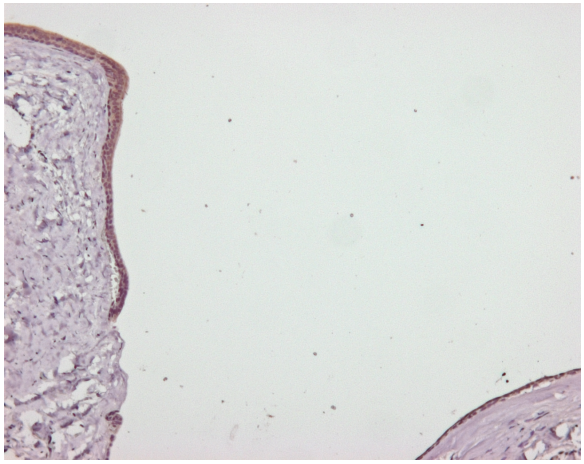
Keratocystic Odontogenic Tumour



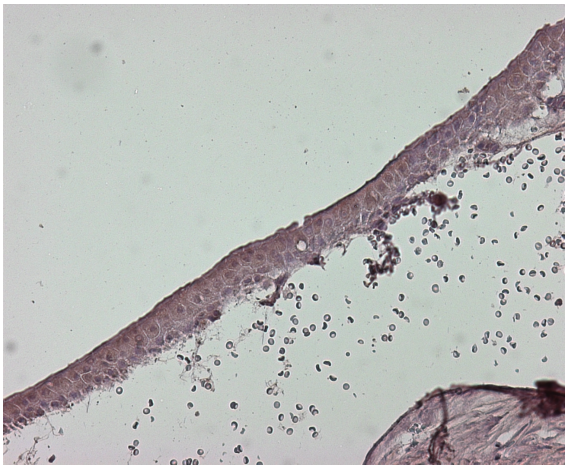
Dentigerous Cyst



Lateral Periodontal Cyst

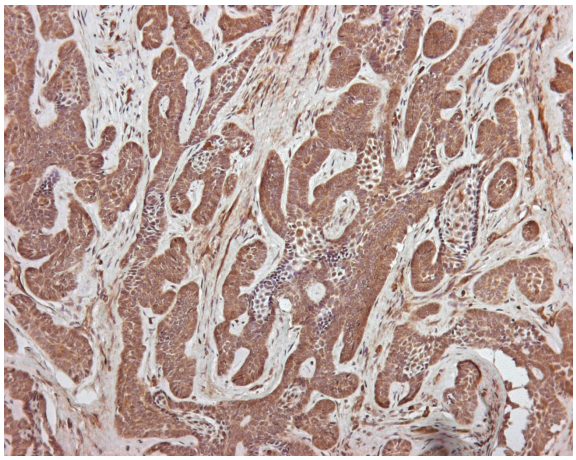


Nasopalatine Duct Cyst (50x)

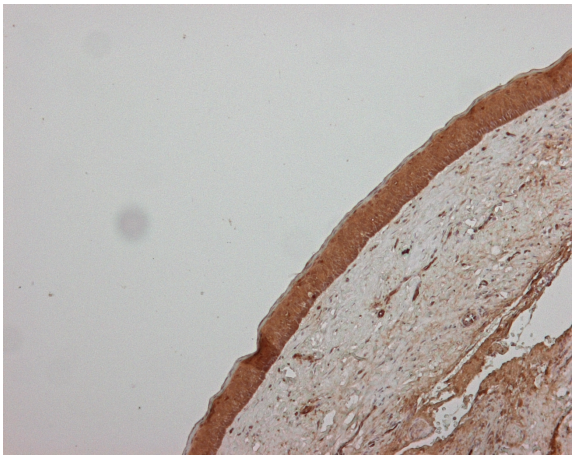


Odontoma

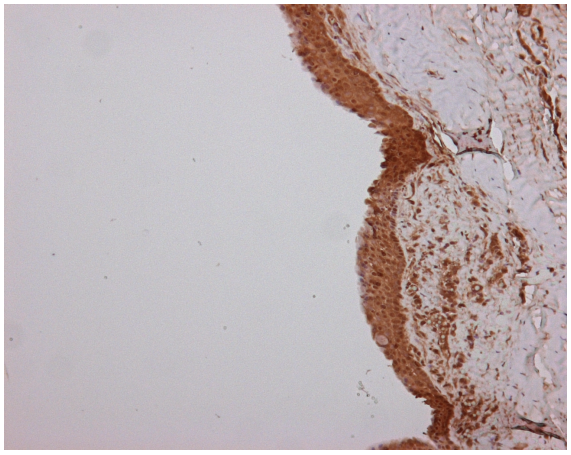
Figure 6.2 - KLK 4 Immunostaining (indicated by brown stain in tissues)



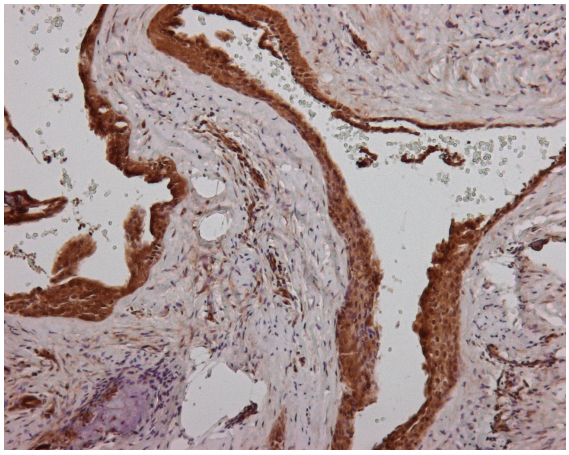
Ameloblastoma



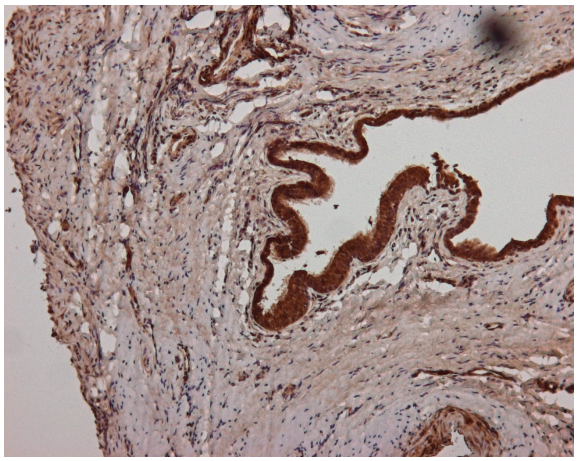
Keratocystic Odontogenic Tumour



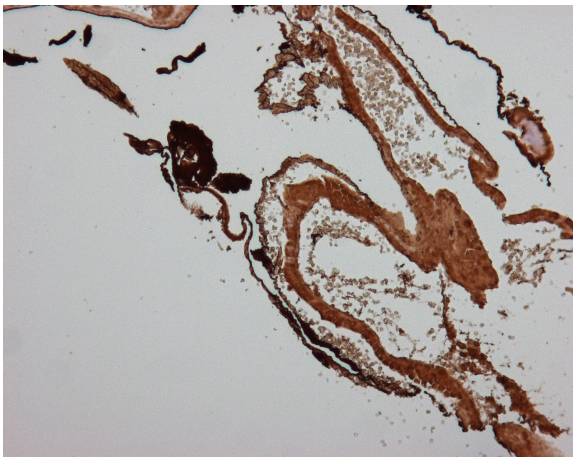
Dentigerous Cyst



Lateral Periodontal Cyst

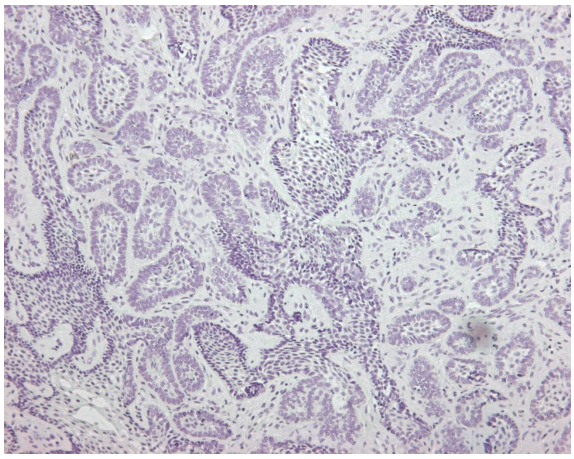


Nasopalatine Duct Cyst (50x)

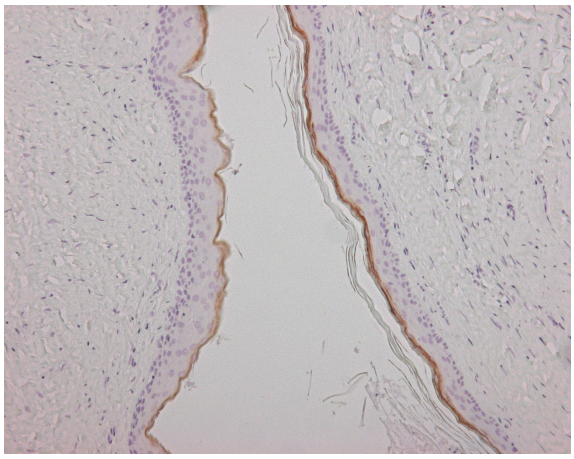


Odontoma (50x)

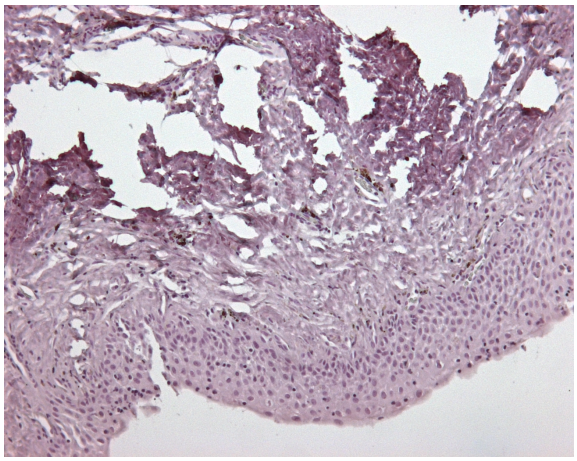
Figure 6.3 - KLK 5 Immunostaining (indicated by brown stain in tissues)



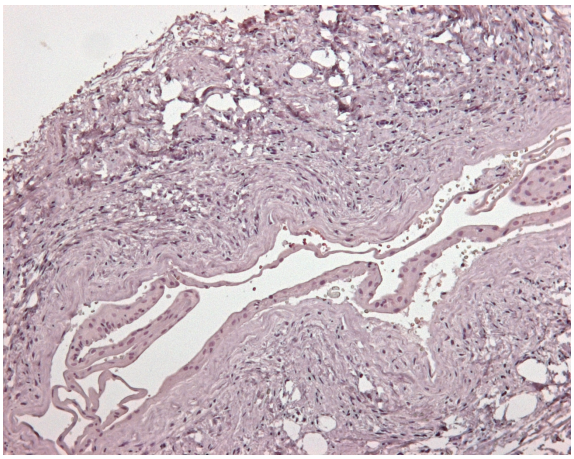
Ameloblastoma



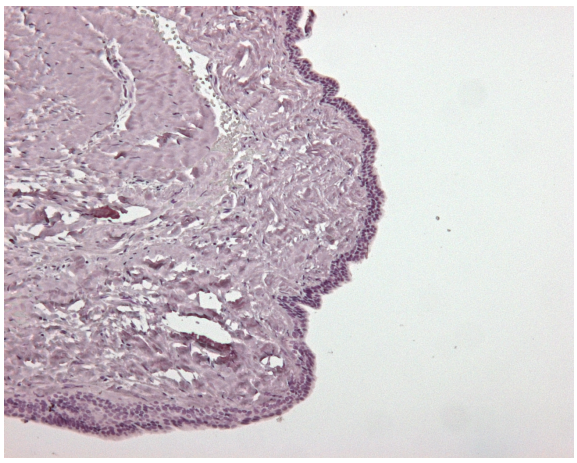
Keratocystic Odontogenic Tumour



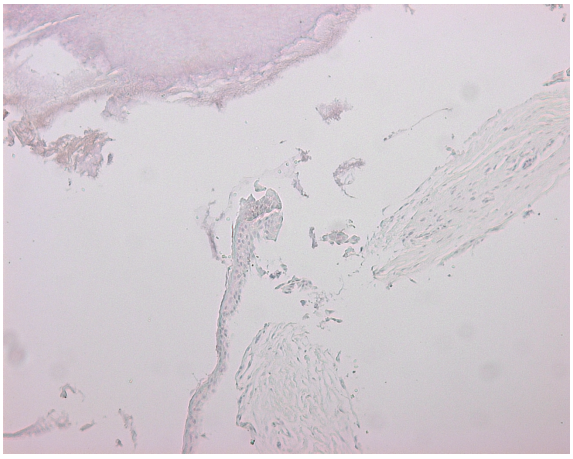
Dentigerous Cyst



Lateral Periodontal Cyst

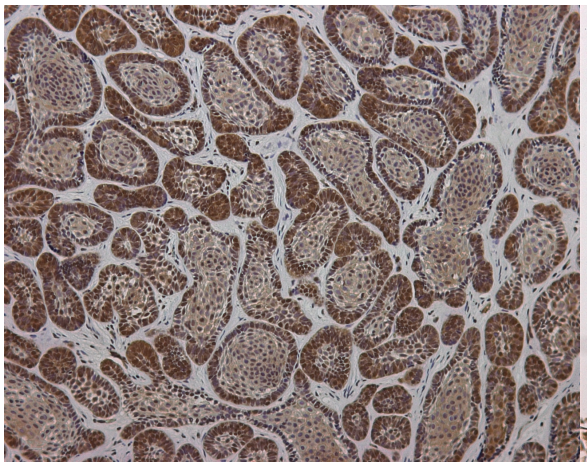


Nasopalatine Duct Cyst (50x)

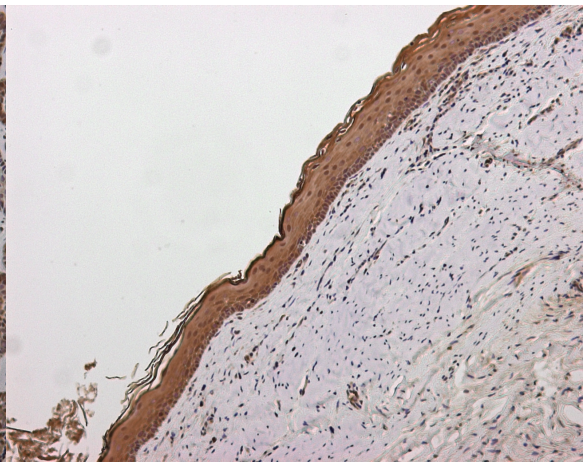


Odontoma (50x)

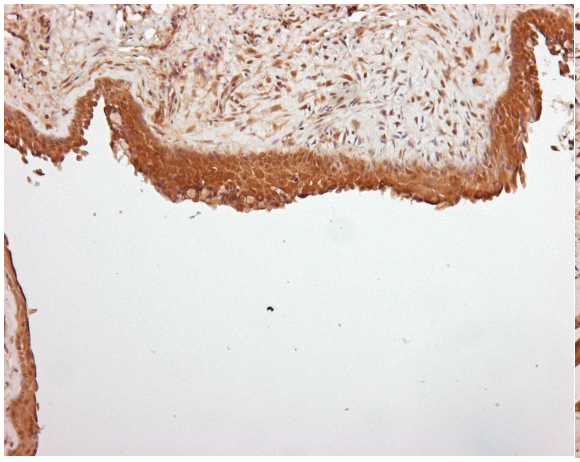
Figure 6.4 - KLK 9 Immunostaining (indicated by brown stain in tissues)



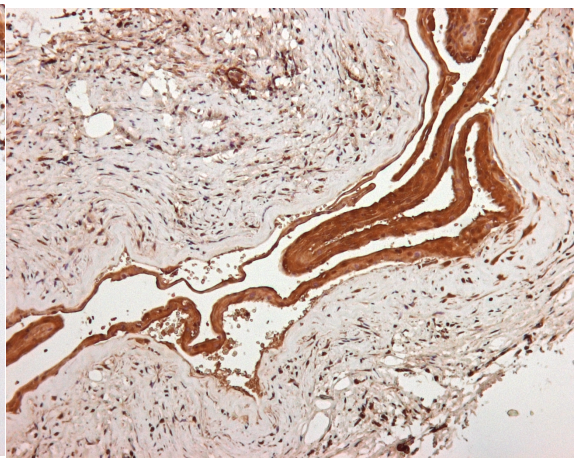
Ameloblastoma



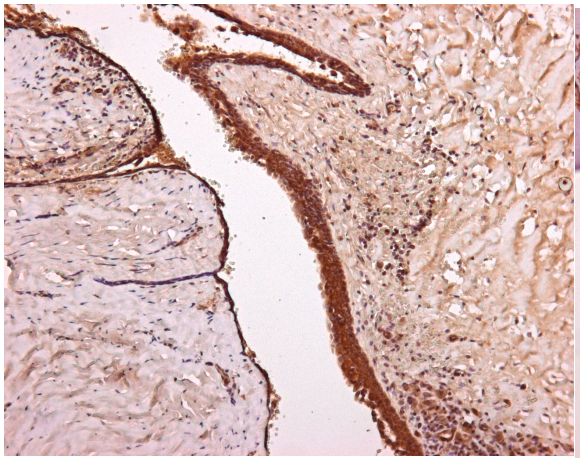
Keratocystic Odontogenic Tumour



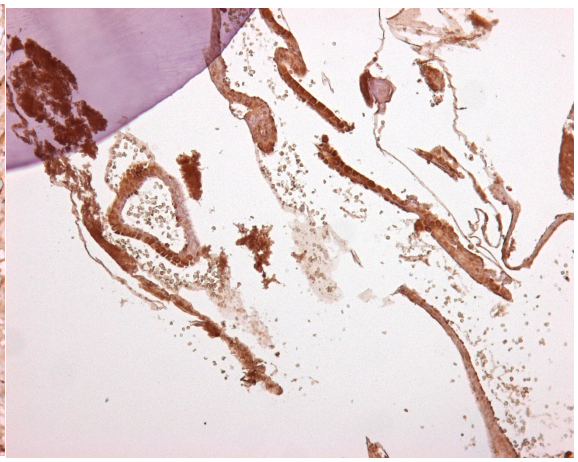
Dentigerous Cyst



Lateral Periodontal Cyst

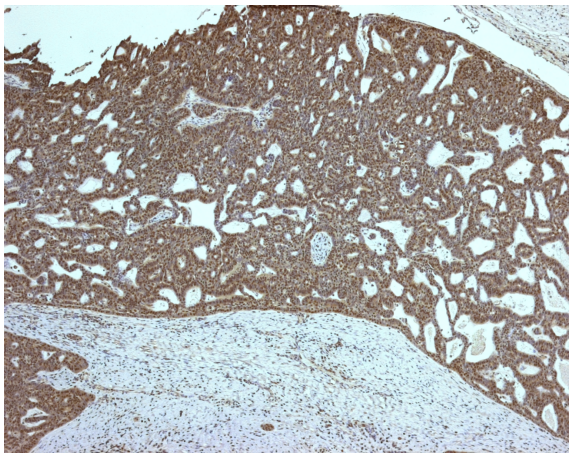


Nasopalatine Duct Cyst (50x)

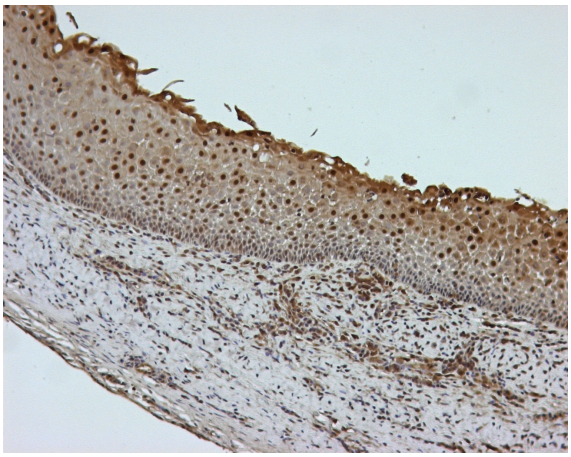


Odontoma (50x)

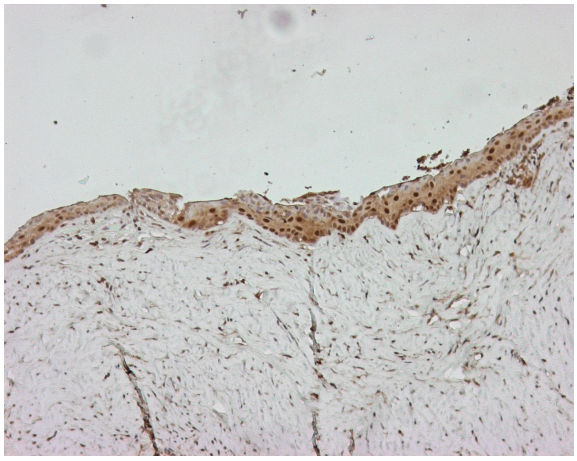
Figure 6.5 - KLK 11 Immunostaining (indicated in brown stain in tissues)



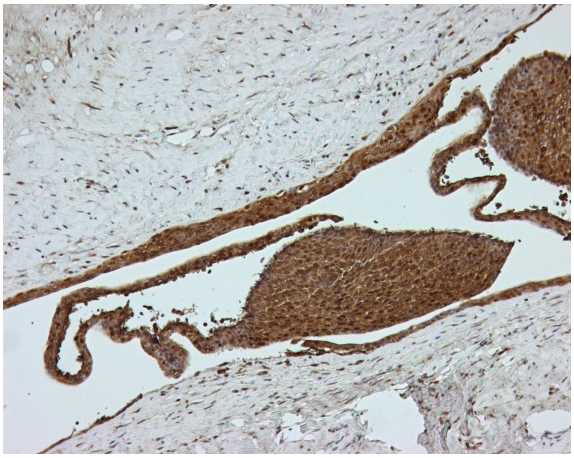
Ameloblastoma (50x)



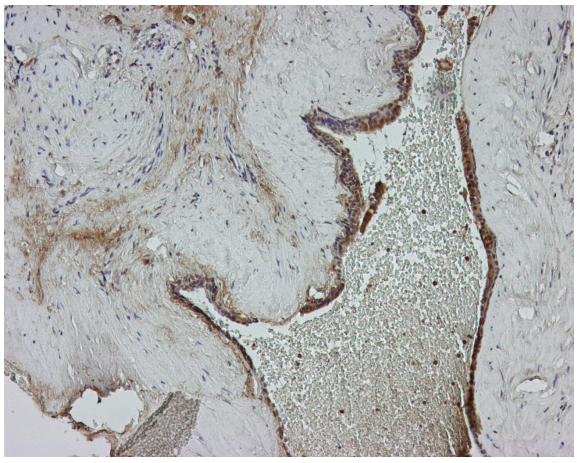
Keratocystic Odontogenic Tumour



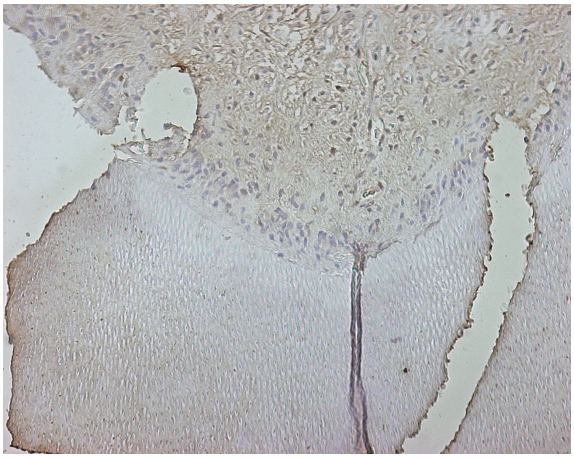
Dentigerous Cyst



Lateral Periodontal Cyst

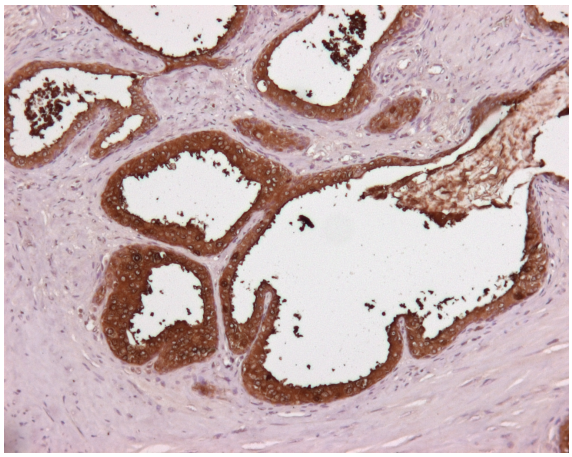


Nasopalatine Duct Cyst (50x)

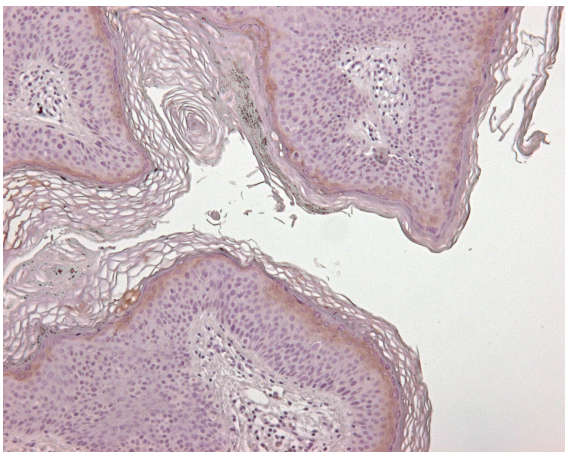


Odontoma

Figure 6.6 - Control Immunostaining (indicated by brown stain in tissues)



Prostate (Control for KLK 3, 4, 9, 11) (50x)



Sebhorrheic Keratosis (Control for KLK 5)

Table 6.1 - Mean Values (+/- Standard Deviation) for Staining Intensity in Odontogenic Cysts, Tumours and Controls

Tissue Type	KLK 3	KLK 4	KLK 5	KLK 9	KLK 11
Lateral Periodontal Cyst	5.6 +/- 2.2	7.3 +/- 0.9	0	7.8 +/- 0.5	6.3 +/- 2.7
Dentigerous Cyst	4.9 +/- 2.6	7.4 +/- 0.5	0	7.9 +/- 0.4	6.9 +/- 0.6
Keratocystic Odontogenic Tumour	6.0 +/- 0.6	7.5 +/- 0.5	2.7 +/- 2.2	7.7 +/- 0.5	6.6 +/- 0.5
Ameloblastoma	6.5 +/- 0.7	7.5 +/- 0.7	0	6.9 +/- 0.3	7.3 +/- 0.6
Odontoma	3.5 +/- 2.7	6.1 +/- 3.4	0	6.3 +/- 2.2	5.1 +/- 2.7
Nasopalatine Duct Cyst	5.4 +/- 0.7	7.6 +/- 0.5	0	7.3 +/- 0.8	5.7 +/- 1.1

Table 6.2 - Median Values for Staining Intensity in Odontogenic Cysts, Tumours and Controls

Tissue Type	KLK 3	KLK 4	KLK 5	KLK 9	KLK 11
Lateral Periodontal Cyst	6	8	0	8	7
Dentigerous Cyst	6	7	0	8	7
Keratocystic Odontogenic Tumour	6	7	4	8	7
Ameloblastoma	7	8	0	7	7
Odontoma	4	8	0	7	6
Nasopalatine Duct Cyst	6	8	0	7.5	5

Figure 6.7 - Mean Immunostaining Score Illustrated for Each Tissue Type Studied

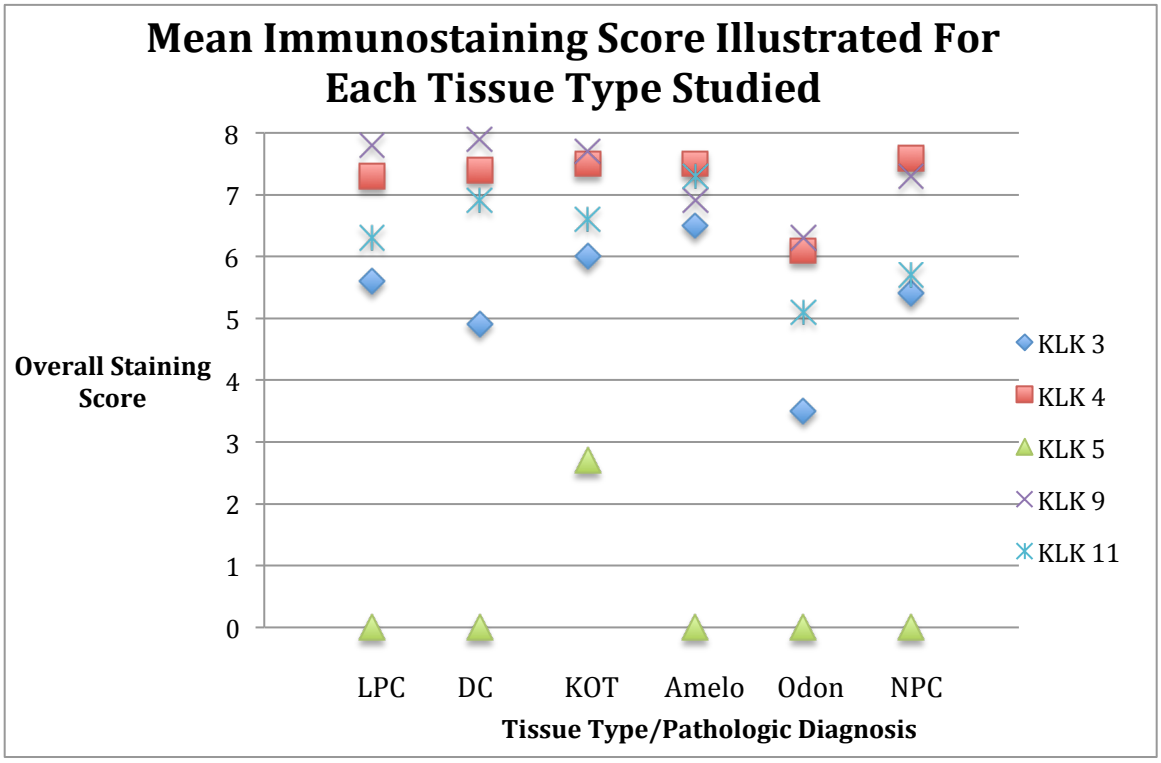


Figure 6.8 - Mean Immunostaining Score Illustrated for Each KLK Investigated

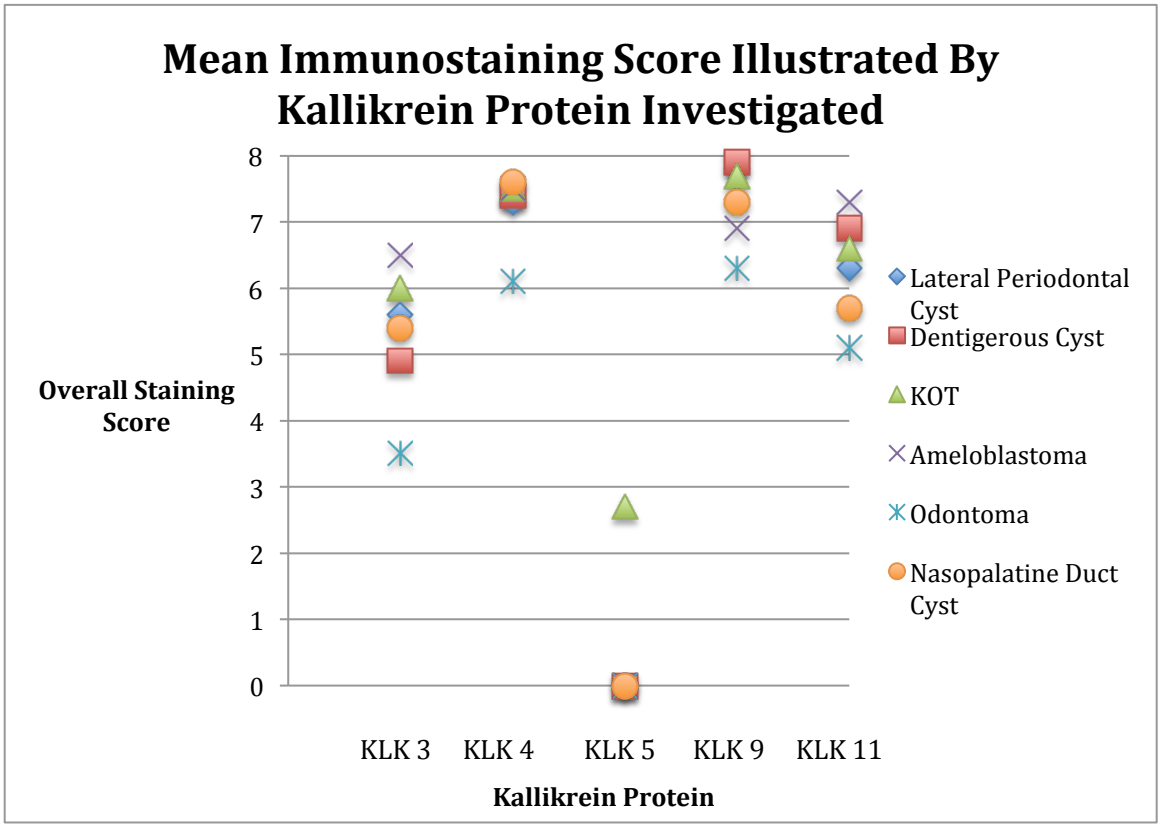


Figure 6.9 - Median Immunostaining Score Illustrated for Each Tissue Type Studied

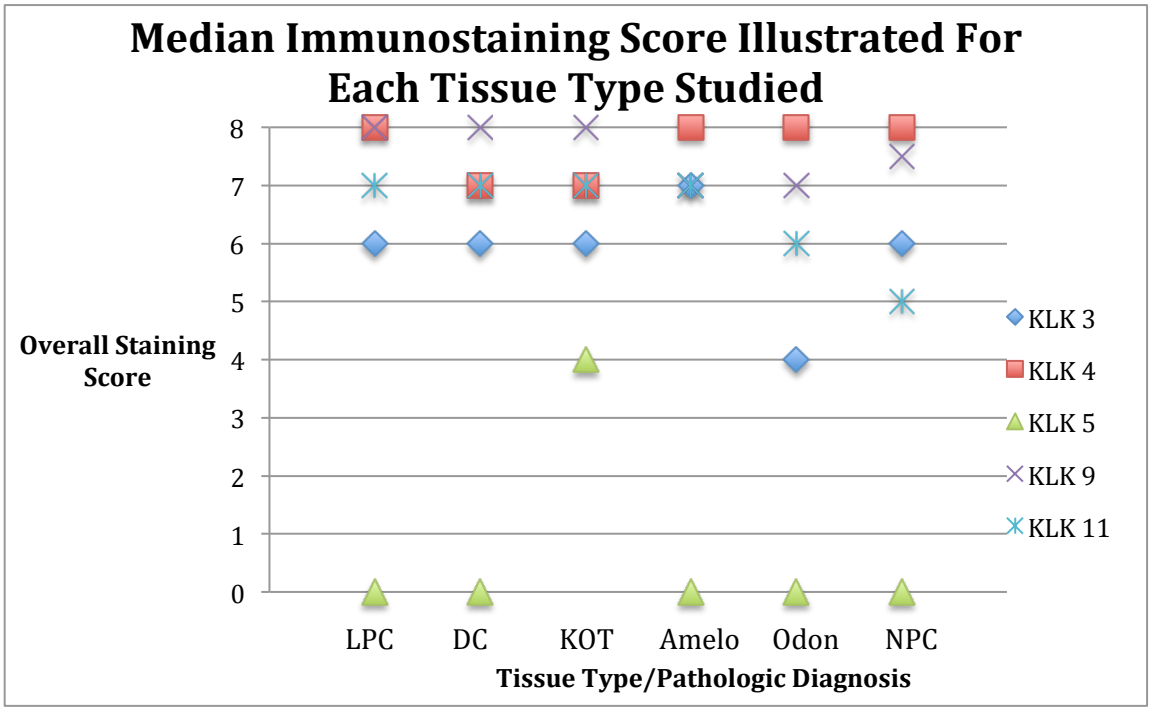
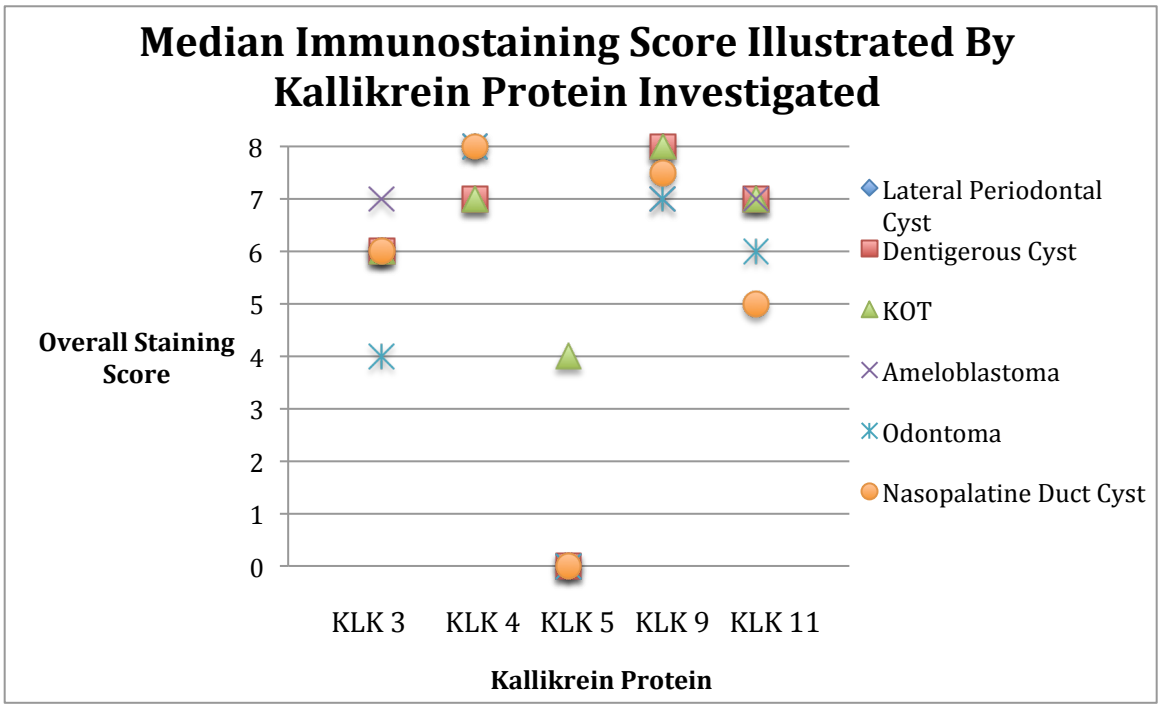


Figure 6.10 - Median Immunostaining Score Illustrated for Each Tissue Type Studied



6.1.1 Immunostaining Statistical Analysis

KLK 3, 4, 9 and 11 are identified through immunostaining in the Ameloblastoma, KOT, Dentigerous Cyst, Lateral Periodontal Cyst, Odontoma and Nasopalatine Duct Cyst. KLK 5 is only present in the KOT.

For KLK 3, the Kruskal-Wallis test reveals a p value of 0.004, showing significant variance between the lesions investigated. The Dunn's multiple comparison test shows significantly more staining in ameloblastoma tissue than to odontoma ($p<0.01$) and KOT than odontoma ($p<0.05$). No other statistically significant differences were noted. No other statistically significant differences were identified.

Analysis of KLK 4 staining using the Kruskal-Wallis test reveals a p value of 0.5554, signifying no difference between groups investigated.

For KLK 5 the Kruskal-Wallis test revealed a p value of < 0.0001, identifying that variation amongst the lesions is greater than can be expected by chance. Dunn's multiple comparison test reveals that $p < 0.001$ showing that KLK 5 was statistically significantly more prevalent in the KOT than all other lesions investigated.

Analysis of KLK 9 staining using the Kruskal-Wallis test reveals a p value of 0.0002. This statistically significant difference was further evaluated using the Dunn's multiple comparison test which revealed more KLK 9 staining in the Dentigerous Cyst than Odontoma ($p<0.05$), KOT than Odontoma ($p<0.05$), Lateral Periodontal Cyst than Ameloblastoma ($p<0.05$), Dentigerous Cyst than Ameloblastoma ($p<0.01$), and KOT than Ameloblastoma ($p<0.05$). No other statistically significant differences were identified.

Analysis of KLK 11 staining using the Kruskal-Wallis test reveals a p value of 0.0025, showing that there is statistically significant variance amongst the study groups. Dunn's multiple comparison test reveals $p<0.05$ when comparing KLK 11 staining in the Ameloblastoma and the Nasopalatine Duct Cyst; more staining is present in the Ameloblastoma than the Nasopalatine Dyct Cyst. No other statistically significant differences were identified.

Table 6.3 – Statistically Significant Differences in Kallikrein Immunostaining

Kallikrein Protein	Kruskal-Wallis (Analysis of Variance)	Dunn's Multiple Comparison Test
KLK 3	P = 0.0040	Ameloblastoma > Odontoma, p<0.01 KOT > Odontoma, p<0.05
KLK 4	P = 0.5554	No Difference
KLK 5	P < 0.0001	KOT > Odontoma, p<0.001 KOT > Nasopalatine Duct Cyst, p<0.001 KOT > Ameloblastoma, p<0.001 KOT > Dentigerous Cyst, p<0.001 KOT > Lateral Periodontal Cyst, p<0.001
KLK 9	P = 0.0002	Dentigerous Cyst > Odontoma, p<0.05 Keratocystic Odontogenic Tumour > Odontoma, p<0.05 Lateral Periodontal Cyst > Ameloblastoma, p<0.05 Dentigerous Cyst > Ameloblastoma, p<0.01 Keratocystic Odontogenic Tumour > Ameloblastoma, p<0.05
KLK 11	P = 0.0025	Ameloblastoma > Nasopalatine Duct Cyst, p<0.05

6.2 mRNA Analysis

KLK 1, 4, 7, 8, 10 and *12* genes were identified using PCR for pooled ameloblastoma tissue from nine of the ten tissue samples used for immunostaining. Insufficient cDNA was produced to facilitate use for RT-PCR from the tenth ameloblastoma tissue sample. *KLK 1-15* were assessed using RT-PCR. *KLK 2, 3, 5, 6, 9, 11, 13, 14* and *15* were not detected using the current study methods. Results were calculated using the difference in cycle threshold (CT) (Table 6.4 and 6.5) as well as standard curve (Table 6.6 and 6.7) methods of analysis. Using the standard curve method, average relative mRNA levels were detected, and are depicted with associated standard deviation in Figure 6.11.

Table 6.4 – Raw PCR Data – Run 1 Change in CT Method

Gene	Ct 1	Ct 2	Ct Average	Concentration	Relative mRNA Levels
KLK 1	48.81	51.97	50.39	19.10015	0.0000018
KLK 4	49.81	49.61	49.71	18.42137	0.0000028
KLK 7	39.81	39.71	39.76	8.47041	0.0028194
KLK 8	41.28	44.99	43.13	11.84467	0.0002719
KLK 10	32.72	32.36	32.54	1.25107	0.420136
KLK 12	43.35	42.59	42.97	11.68106	0.0003045
Beta-Actin	31.38	31.19	31.29	NA	NA

Table 6.5 - Raw PCR Data – Run 2 Change in CT Method

Gene	Ct 1	Ct 2	Ct Average	Concentration	Relative mRNA Levels
KLK 1	41.88	45.91	43.89	14.88492	0.0000331
KLK 4	44.73	43.64	44.19	15.17644	0.0000270
KLK 7	38.25	37.24	37.74	8.73434	0.002348
KLK 8	43.24	43.49	43.37	14.35506	0.0000477
KLK 10	32.19	31.01	31.60	2.59312	0.165727
KLK 12	42.81	42.31	42.56	13.55115	0.000083
Beta-Actin	29.01	29.00	29.01	NA	NA

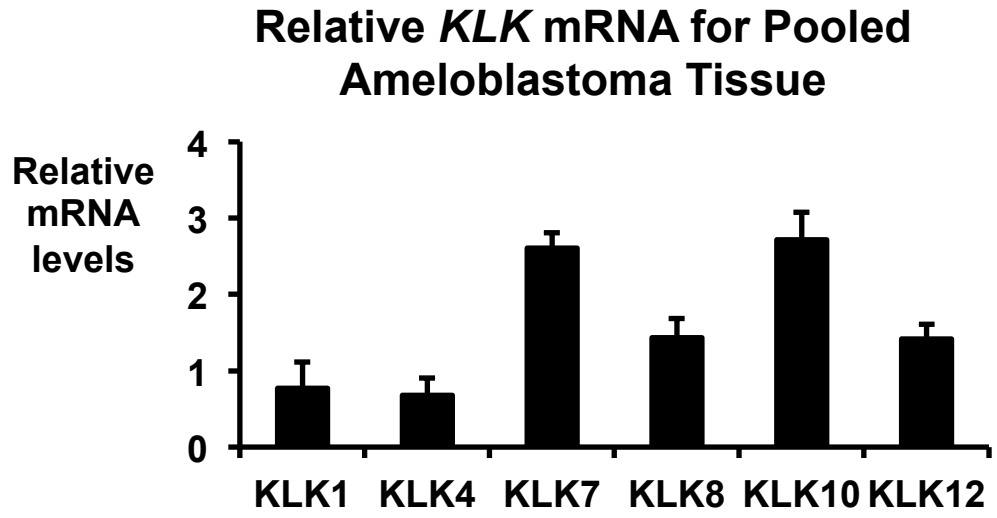
Table 6.6 - Raw PCR Data – Run 2 Standard Curve Method

Gene	Ct 1	Ct 2	Ct Average	Concentration	Relative mRNA Levels
KLK 1	41.88	45.91	43.89	23.38405	1.01015
KLK 4	44.73	43.64	44.19	19.36874	0.83669
KLK 7	38.25	37.24	37.74	57.08126	2.46581
KLK 8	43.24	43.49	43.37	28.97867	1.25183
KLK 10	32.19	31.01	31.60	56.78855	2.45316
KLK 12	42.81	42.31	42.56	29.46897	1.27300
Beta-Actin	29.01	29.00	29.01	23.14909	NA

Table 6.7 - Average Relative mRNA Levels

Gene	Relative mRNA Average	Standard Deviation
KLK 1	0.76961	0.34017
KLK 4	0.67650	0.22655
KLK 7	2.60676	0.19934
KLK 8	1.43129	0.25380
KLK 10	2.71217	0.36629
KLK 12	1.41314	0.19818

Figure 6.11 - Relative Expression of KLK mRNA for KLK's Detected in Pooled Ameloblastoma Tissue



Chapter 7

7 Discussion

7.1 Expression of KLK 3, 4, 5, 9 and 11 in Maxillofacial Cysts and Tumours

For the first time, Kallikrein Protein 3, 4, 5, 9 and 11 have been identified in oral and maxillofacial cysts and tumours. KLK 3, 4, 9 and 11 were identified in odontogenic and non-odontogenic cysts and tumours, while KLK 5 was identified only in the KOT. Prior research from our lab has identified the presence of KLK 6, 7, 8, 10, 13 and 14 in odontogenic cysts and tumours (58).

KLK 3 staining was significantly greater in the benign neoplasms of odontogenic origin, the Ameloblastoma and KOT compared to odontogenic control, the Odontoma. In the Ameloblastoma tissue, there was patchy staining of the connective tissue stroma, consistent with KLK 3 being a secreted protein. Macrophage staining and stromal staining were also present in the tissue surrounding the KOT. In the Odontoma specimens, care was taken to ensure that staining was only counted when odontogenic epithelial cells were visualized, as uniformly intense staining of enamel protein was present in some specimens. The unique increased expression in the benign odontogenic neoplasms (Ameloblastoma and KOT) suggests that KLK 3 may have a role in the intrinsic and limitless growth potential of these lesions compared to less clinically aggressive odontogenic cysts. KLK 3 has been identified to be involved in tumour invasion by direct extracellular matrix degradation as well as tumour activation by TGF-Beta and Insulin like growth factor (21). It may be that KLK 3 is involved in the growth of these lesions through the aforementioned mechanisms and signaling cascades, or through alternative mechanisms not yet identified. In the second series of experiments as part of this thesis, *KLK 3* mRNA was not identified in the Ameloblastoma when utilizing RT-PCR for the pooled Ameloblastoma tissue. This suggests that KLK 3 protein may be recruited to the Ameloblastoma where it was stained and identified microscopically. It may similarly be recruited to the KOT, and/or other odontogenic lesions staining positively for KLK 3, or the *KLK 3* gene may actually be expressed in these

tumours/cysts, resulting in intrinsic Kallikrein protein production. Alternatively, KLK 3 may have a very long half life, allowing it to be identified within the lesional tissue stained while not identified using the RT-PCR assay utilized in this study due to minimal abundance of the mRNA. Nonetheless, the presence of statistically significantly increased KLK 3 protein in these tumours would be interesting to further investigate. Studying this protein may contribute to an understanding of the more aggressive behaviour observed in the Ameloblastoma and KOT compared to odontogenic cysts. Our data and experimental design do not readily support any specific mechanism of action of KLK 3, however potential roles could include KLK 3 acting as a part of a larger cascade of molecular signaling involving other KLK proteins where it may function to permit local tissue destruction resulting in extension/invasion of the lesion, or act in a cascade as an activator of growth or as an inhibitor of growth suppression. The relatively wide variety of physiologic processes and pathways of molecular signaling identified to be associated with KLKs suggest that KLK 3 could be involved in the pathogenesis of these lesions in many possible ways.

KLK 3 has been widely studied with respect to its role in urologic physiology, and is known as a biomarker in prostate cancer. Levels of prostate specific antigen (KLK 3) are measured utilizing a serum assay, and followed in cancer surveillance. It would be interesting to study the plasma levels of prostate specific antigen in individuals with odontogenic cysts or tumours in women or men with no known prostate disease to see if there are increased levels of serum PSA in these individuals. If PSA/KLK 3 was detected in the serum of patients with odontogenic pathology, it may be prudent to screen individuals being followed for prostate cancer for odontogenic pathology to ensure it does not alter the post-cancer surveillance for these patients.

KLK 4 staining was prevalent in all study and control tissue. No significant difference was noted between any of the groups analyzed. It was expected that KLK 4 would be present in all odontogenic tissues (Ameloblastoma, KOT, Dentigerous Cyst, Lateral Periodontal Cyst and Odontoma) due to the known role of KLK 4 in amelogenesis. The Nasopalatine Duct Cyst (non-odontogenic maxillofacial cyst) surprisingly exhibited KLK 4 immunostaining with similar magnitude to that observed in

the odontogenic tissues. In addition to its role in amelogenesis, KLK 4 has been implicated in the activation of urokinase type plasminogen activator which can promote tumour invasion through its action. In non-odontogenic tissue such as the Nasopalatine Duct Cyst, KLK 4 may have a role in a signaling cascade involving other proteins, possibly including other KLKs, or may be recruited to the site of the lesion and sequestered. Qualitative observations made in the histopathologic analysis and scoring include the fact that the Ameloblastoma tissue exhibits minimal staining in the stellate reticulum compared to the cytoplasm of the lesional Ameloblasts and that enamel stains significantly while dentin exhibits no staining in the Odontoma tissues when investigating for KLK 4. As outlined previously, this was expected due to the known role of KLK 4 in normal enamel maturation. As expected, KLK 4 mRNA was identified using RT-PCR in the pooled Ameloblastoma tissue. It would be interesting to study odontogenic and non-odontogenic pathologic lesions in patients with known KLK 4 mutation. While these individuals would be expected to have opaque, abnormal enamel deposition, it would be interesting to see if KLK 4 was still recruited to the tumour/cyst tissue, where it may function in an additional signaling cascade with proteolytic functions apart from those in normal amelogenesis.

KLK 5 was only identified in the KOT. The KLK 5 staining was localized to the parakeratin layer within the KOT and no KLK 5 staining was present in any of the remaining tissue types investigated. It was not surprising to find KLK 5 localized to the parakeratin of the KOT, as this parakeratinized cyst was the only pathology to be investigated with keratin maturation. Despite the fact that no other tumours or cysts identified produce a keratin layer, we were surprised that KLK 5 was not identified in any other tissue types investigated as KLK 5 has been identified to be capable of autoactivation, and is involved in multiple KLK signalling cascades. KLK 5 is identified in the granular cell layer of the epithelium of the skin; therefore Seborrheic Keratosis was utilized as a control tissue for staining. KLK 5's role in the antimicrobial activity of skin, normal epithelial turnover and in dermatologic disorders such as peeling skin syndrome suggests that it is required for normal turnover of keratinized epithelium. It would be interesting to investigate the Orthokeratinized Odontogenic Cyst for the presence of KLK 5, as it has similar histopathologic findings as the KOT, but without the parakeratin layer.

It would also be beneficial to complete RT-PCR on KOT specimens in order to investigate whether KLK 5 is produced intrinsically by the KOT/epithelium or whether it is recruited to this site where it can then function in the normal desquamation of the parakeratinized epithelium. In odontogenic tumours and cysts, it is unlikely that KLK 5 is involved in signaling cascades regulating tumour/cyst growth and behaviour due to the fact that it is only expressed in the keratinized tumour, the KOT.

KLK 9 staining was present in all tissues studied. Significantly increased staining was present for the Dentigerous Cyst compared to the Odontoma (odontogenic control) and Ameloblastoma. Significantly increased staining was present in the KOT when compared to the Odontoma and Ameloblastoma. The Lateral Periodontal Cyst also exhibited significantly increased staining when compared to the Ameloblastoma. Nuclear and cytoplasmic staining was noted in the Ameloblastoma tissue when staining for KLK 9. In the KOT specimens, generalized subepithelial staining was identified. Patchy staining was visualized in the basal layer of the Nasopalatine Duct Cyst tissue in a number of tissue specimens. Staining for KLK 9 showed great variability in the Odontoma tissues. Enamel matrix stained profoundly with KLK 9 in Odontoma tissue. This immunostaining shows a general trend for increased staining of KLK 9 in odontogenic cysts (Dentigerous Cyst, Lateral Periodontal Cyst) or cystic neoplasms (KOT) compared to the solid, most clinically aggressive odontogenic neoplasm (Ameloblastoma) or odontogenic hamartoma (Odontoma). KLK 9 has been identified as a favourable prognostic marker in breast and ovarian cancer, and may once again be a favourable marker for odontogenic pathologies (18, 21, 23) as it is less abundant in the more aggressive ameloblastoma. The role of KLK 9, and its exact mechanism of action conferring favourable prognosis in the aforementioned malignancies is not yet identified, and could be investigated with respect to the specific pathogenesis of odontogenic cysts/tumours. Clinical treatment of the Dentigerous Cyst and Lateral Periodontal Cyst is simple enucleation; low rates of recurrence are achieved with this treatment modality (5-7, 11). Treatment for the benign cystic neoplasm, the KOT is controversial, but en-bloc resection with a margin of unaffected tissue is not indicated as a first line of therapy as it is in management of the Ameloblastoma (5-7, 11). The Ameloblastoma is known to have high rates of recurrence partly due to tumour cells extending beyond the radiographically

visualized margin of the lesion. Malignancies can invade, fragment and metastasize, all features correlated with worse prognosis. If KLK 9 is involved in the prevention of budding or fragmentation of tumours, it could explain a possible role of KLK 9 based on its pattern of identification in our study, and those identifying it as a favourable marker in breast and ovarian malignancy.

KLK 9 was not identified in the Ameloblastoma when investigating mRNA expression using RT-PCR. In order to investigate the expression and localization of KLK 9, RT-PCR would once again be useful to investigate the less clinically aggressive odontogenic cysts/tumours, the Dentigerous Cyst, Lateral Periodontal Cyst and KOT for the presence and abundance of *KLK 9*. If not present, KLK 9 protein may be recruited to these lesions where it may function in a regulatory cascade. Correlation of the natural history/clinical manifestation of many Ameloblastomas of variable clinical course (i.e. Unicystic Ameloblastoma vs Solid Ameloblastoma vs Peripheral Ameloblastoma) with the individual tumour's specific KLK 9 expression could help to determine if KLK 9 is a positive prognostic indicator in this specific lesion. If higher abundance of KLK 9 expression in Ameloblastoma tissue could be identified on initial incisional biopsy, and research indicated that higher expression of KLK 9 confers favourable prognosis (less likely to recur), it may be possible to tailor surgical therapy to the unique biology of a patient's tumour. At the present time, this is only a theoretical consideration; studies with more power, specific clinical correlation and long term follow up would be required in order to make this a potential reality.

KLK 11 staining was identified in all six tissue types investigated in this study. KLK 11 staining was significantly greater in the Ameloblastoma than in the non-odontogenic control, the Nasopalatine Duct Cyst. In the KOT, well-formed keratin stained intensely when desquamating, while in specimens with no visible desquamating keratin, immunostaining was less intense. This qualitative finding that KLK 11 staining was more intense in KOT lesions with visible desquamating keratin suggests that KLK 11 may have a role in the epithelial turnover, and/or that it may be sequestered in desquamated keratin once these cells desmosomal attachment is lost. It may function in conjunction with KLK 5 as part of this process. Desmosomal staining was visualized

with staining of the Dentigerous Cyst. The Dentigerous Cysts also exhibited intense nuclear staining of epithelial cells. The enamel matrix of Odontoma stained intensely for KLK 11. KLK 11 is known to be present in non-malignant prostate tissue, is involved in the desquamation of vaginal epithelium and is a favourable prognostic marker in ovarian cancer. The nonspecific identification of KLK 11 in all six tissue types studied suggests it may be involved in normal cell turnover, or ubiquitous processes within cells of ectomesenochymal origin.

Kallikrein proteins have been utilized as biomarkers, most notably in the monitoring and surveillance of prostate cancer. A more complete review of their roles as biomarkers is provided in the introduction of this thesis. Kallikrein proteins have not yet been identified as biomarkers in pathology of the head and neck. Due to the diverse array of odontogenic pathologies that present in the maxillofacial region, as well as the relatively similar clinical and radiographic findings (especially in the early stages of growth) of lesions with variable clinical behaviour/natural history, tissue biopsy is recommended prior to definitive surgical management of these lesions.

In addition to identifying biomarkers in serum, saliva is another non-invasive source of systemic proteins for use in clinical decision making (59-61). Quantification of KLK protein in saliva or serum could potentially act as an adjunct, or sole pre-operative test prior to the definitive surgical management of various odontogenic pathologies. This could allow a patient to undergo the appropriate operative intervention without having a surgical incisional biopsy of the intraosseous lesion of interest. The ability to identify easily accessible biomarkers for these lesions would be especially useful in populations of individuals without access to surgical consultation. In Canada, this would include individuals geographically isolated such as those in remote northern communities. Worldwide, this would involve many groups of people in non-industrialized areas without easy access to comprehensive medical care. If a relatively indolent process such as the lateral periodontal cyst was identified based on biomarker analysis, it may not be in the patient's best interest to travel a significant distance in order to have formal biopsy, or removal of the lesion, while if an aggressive lesion such as an ameloblastoma was identified, it would be worth seeking surgical consultation.

In addition to their role in identifying specific diseases/lesions, the presence or absence of specific biomarkers may confer a positive or negative prognosis. It could be possible that biomarkers (such as KLKs) may be identified in the Ameloblastoma or KOT that predict their likelihood for recurrence. With this information, the surgical intervention could be individualized for the specific pathology in each individual; the generation of databases with proteomic profiles could be used to provide personalized medicine to patient's with these lesions.

While our methodology does not investigate the KLK proteins for their potential role as biomarkers, it does provide the first step required to consider further investigation. The pattern of expression of *KLK* genes and KLK protein identified in this data suggest that low levels of KLK 9 are correlated with more aggressive odontogenic pathology (the Ameloblastoma). The presence of KLK 5 in serum or saliva in the context of an odontogenic lesion would possibly be indicative of a KOT due to its unique expression in the parakeratin layer of the KOT and not in other odontogenic tumours/cysts. The identification of KLK 3 protein/staining in benign odontogenic neoplasms in greater amounts than in less clinically aggressive cysts could possibly provide another usage of KLKs as biomarkers, as identification of KLK 3 may narrow a differential diagnosis to one of these more aggressive lesions when compared to lesions with less expression of KLK 3.

While there are a number of theoretical links based on the preliminary research outlined in this thesis, a significant amount of further research would be required to consider using KLKs as biomarkers for odontogenic cysts and tumours. Due to the ubiquitous nature of KLK proteins in multiple organ systems, it may not materialize as a clinically relevant investigation even with differential expression of KLK proteins within the various odontogenic pathologies. It seems that the most likely utilization of differential quantification of KLK proteins/analysis of the relative abundance of KLK would be in determining the relative propensity for a specific patient's tumour/cyst to recur or grow. This could be based on KLK profile using serum or salivary fluid to evaluate biomarkers, or through the use of immunostaining in histopathologic diagnosis.

Immunohistochemistry is an appropriate method to investigate tissue for the presence of protein, but is not without limitations. The analysis used is a validated subjective assessment (25, 49, 50, 58) requiring the input from experienced independent observers to quantify the magnitude of staining. In the process of analysis of the immunostained slides, we attempted to use a computer based analysis of staining intensity in order to provide more objective data on the staining intensity. Unfortunately, due to the minimal volume of tissue to be analyzed (i.e. a single or double cell layer of enamel epithelium around odontomas, or 2-5 cell thick Lateral Periodontal Cyst) or varying, unpredictable location of cells of interest (Ameloblasts in various patterns of Ameloblastomas), it was extremely time consuming and not easy to outline the tissue to be analyzed while tissue remained on the slide. Additionally, special care must be taken to be consistent in the specific tissue and cell types (i.e. pre-ameloblasts, clear cells, enamel, dentin) included and excluded in the analysis to prevent error in the interpretation from inconsistent comparison between tissue types. As a result of these technical difficulties, we abandoned the idea of using a computer-based assessment of immunostaining.

Another limitation of our study design was the limited abundance of odontogenic epithelium in Odontoma specimens. This may have contributed to the larger standard deviation found within the overall staining score for the Odontoma compared to other tissue types where odontogenic or non-odontogenic epithelium is more abundant. To maximize the volume of normal odontogenic epithelium (i.e. not associated with a pathologic condition derived from the odontogenic epithelium) for study purposes, developing dental follicles taken from aborted fetuses could be used as an odontogenic control. Due to ethical concerns, we decided that it would be appropriate to use Odontoma specimens, as these hamartomas mimic normal odontogenesis and are often associated with adjacent odontogenic epithelium. In the pre-study selection of tissue, we reviewed many Odontoma specimens from the Division of Oral Pathology archives, and made sure to choose Odontoma specimens with some odontogenic epithelium to allow histopathologic analysis of staining.

Using immunohistochemistry to identify and attempt to measure KLK protein, we cannot be certain whether the staining is due to KLK protein sequestered within the tumour/tissue of interest, or if it is actually produced by the tissue/tumour being investigated. While we can make statements about the identification of protein in tissue types, postulate about the utility of KLK protein as a biomarker, or use variable patterns of expression for aid in tissue diagnosis, we cannot make conclusions about the production of these proteins by various tumours/cysts/tissues. As a result, the implications of our findings are less powerful than if investigation about the production of protein, or expression of mRNA was also performed. In order to obtain a more complete understanding of the expression of *KLK* genes, mRNA and production of protein, we can utilize other techniques to investigate for the presence of *KLK* mRNA. Comparing information obtained through the reverse transcription-polymerase chain reaction (to examine for the presence of *KLK* specific mRNA in tissue) with results from immunostaining provides more insight into the role of KLK's in these tumours/cysts of interest. Using RT-PCR we are able to see whether or not the *KLK* genes are active, and can identify specific KLK mRNA in the tissues of interest.

7.2 Expression of Ameloblastoma *KLK* mRNA Using RT-PCR

RT-PCR evaluation of pooled Ameloblastoma tissue revealed the presence of *KLK* mRNA for *KLK* 1, 4, 7, 8, 10 & 12. *KLK* mRNA was not identified for *KLK* 2, 3, 5, 6, 9, 11, 13, 14 or 15. KLK 3, 4, 9 and 11 proteins were identified in Ameloblastoma tissue utilizing immunostaining as described previously in this thesis. Prior work from our laboratory resulted in the identification of KLK 6, 7, 8, 10, 13 and 14 in Ameloblastoma tissue utilizing immunohistochemistry and the identical analysis of immunostaining performed in this study (58). Immunostaining was not performed for KLK 1, 2, 12 and 15 due to unavailability of antibodies to complete the staining procedure. Combining the findings of this study, with those previously identified from our lab, we have identified that KLK 4, 7, 8, and 10 protein and *KLK* 4, 7, 8 and 10 mRNA are present in the Ameloblastoma. *KLK* mRNA was only identified (protein not studied) for *KLK* 1 and 12. KLK protein but not *KLK* mRNA was identified for KLK 3,

6, 9, 11, 13 and 14 when combining the findings of this thesis with prior work from our laboratory (58). The co-identification of KLK protein and *KLK* mRNA strongly suggest that the Ameloblastoma tissue produces KLK 4, 7, 8 and 10 proteins. We cannot comment on the relationship between *KLK* 1 and 12 mRNA and KLK 1 and 12 proteins since no immunostaining was performed for KLK 1 and 12. The identification of the respective KLK protein with immunostaining for all other KLKs with mRNA identified (KLK 4, 7, 8, and 10) in this study suggests that KLK 1 and 12 protein are likely also found in the ameloblastoma tissue.

KLK 3, 6, 9, 11, 13 and 14 may be recruited to the Ameloblastoma, and sequestered in the tumour tissue resulting in the identification of these proteins with immunostaining, while their respective mRNA was not identified using the PCR methodology in this study. Alternatively, the protein may have a long half life and remain in the tissue despite low levels of mRNA expression locally at the time of analysis. KLK 3, 6, 9, 11, 13 and 14 mRNA may be degraded rapidly following production, or may be present in levels below the threshold that can be identified with this method of PCR analysis. In order to evaluate for the presence of *KLK* mRNA where KLK protein was identified but no mRNA identified, a nested PCR analysis could be performed, where *KLKs* are amplified, allowing for greater detection capabilities. Alternatively, the use of fresh tissue samples may provide the ability to identify mRNA not identified in our study. The relative abundance of *KLK* 7 and 10 was higher than that observed for *KLK* 1, 4, 8 and 12, while the overall staining scores for KLK 7 (6.1 +/- 0.9) and KLK 10 (7.5 +/- 0.7) were within the range observed for KLK 4 (7.5 +/- 0.7) and KLK 8 (6.9 +/- 0.3) (58), suggesting that the abundance of mRNA expression does not correlate with magnitude of protein expression. Post-transcriptional modification of mRNA, alterations in translation or post-translational modification or degradation of KLK protein are all possible mechanisms that may account for the absence of a direct relationship between KLK protein and mRNA expression.

Similar to the difficulty described in attempts at utilizing computer based analysis to quantify staining in the small volume of odontogenic tissue of interest with immunostaining, attempts to use a Laser capture microscope to isolate specific

odontogenic epithelium for future use in RT-PCR proved not feasible due to the thin cysts of interest and technical difficulty with the use of the cutting and capturing feature of the microscope. After multiple attempts to use this technique, it was also abandoned in favour of grouping tumours from multiple patient specimens in order to increase the amount of tumour tissue. As a result, pooled Ameloblastoma tissue containing both Ameloblastoma and adjacent tissue from the formalin fixed biopsy specimens were pooled and utilized.

Chapter 8

8 Conclusions

For the first time, human kallikrein protein 3, 4, 5, 9 and 11 have been identified in odontogenic cysts and tumours. In addition, expression of *KLK* 1, 4, 7, 8, 10 and 12 mRNA was identified in the Ameloblastoma.

Specific protein expression patterns were identified utilizing Immunostaining. KLK 3 was identified in all odontogenic cysts and tumours studied, as well as the non-odontogenic control, the developmental maxillofacial cyst, the Nasopalatine Duct Cyst. KLK 3 protein expression was greater in benign neoplasms of odontogenic origin, the Ameloblastoma and KOT compared to odontogenic control, the Odontoma. KLK 4 expression was found in similar magnitude across all odontogenic cysts and tumours investigated, and was also identified in the Nasopalatine Duct Cyst. KLK 5 was only identified in the parakeratin layer of the KOT. No other tissues investigated exhibited KLK 5 expression. KLK 9 was identified in all odontogenic cysts and tumours studied, as well as in the non-odontogenic control, the Nasopalatine Duct Cyst. The Dentigerous Cyst, KOT and Lateral Periodontal Cyst exhibited greater expression of KLK 9 compared to the Ameloblastoma, and the Dentigerous Cyst and KOT exhibited greater expression of KLK 9 compared to the Odontoma. KLK 11 staining was identified in all odontogenic tissue, and the non-odontogenic tissue in this study. KLK 11 staining was significantly greater in the Ameloblastoma than in the non-odontogenic control, the Nasopalatine Duct Cyst.

Co-expression of *KLK* 4, 7, 8 and 10 protein and mRNA was present for the Ameloblastoma. Expression of *KLK* 1 and 12 mRNA was also present in Ameloblastoma tissue.

The ultimate role of KLK protein in odontogenic pathology is not yet identified. For odontogenic pathology, KLK 5 appears to be a specific marker for the KOT. KLK 3 may have a role in the development of more aggressive odontogenic lesions; KLK 9 may be involved in the development of less clinically aggressive odontogenic cysts/tumours.

The relatively ubiquitous expression of KLK protein and KLK mRNA in odontogenic and non-odontogenic tissues is in accordance with past research identifying KLKs in signaling cascades in a wide range of tissues. Their specific roles in each tissue are under investigation, and further investigation of the expression of KLK mRNA and role of KLK protein may provide clues to the regulation and differentiation of odontogenic epithelium in the formation of cysts and tumours. Using this information, it may be possible to develop minimally invasive or medical therapeutic modulators of tumour growth to improve clinical outcomes. If patterns of KLK expression that correlate with the propensity of tumours or cysts to recur could be identified, it may be possible to use this information to perform individualized medicine for patients with these pathologies. Further research is required on these topics.

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